Widerborst Interacts With Bitesize To Regulate Wing Hair Morphogenesis

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I herewith declare that I have produced this work without the prohibited assistance of third parties and without making use of aids other than those specified, notions taken over directly or indirectly from other sources have been identified as such. This work has not previously been presented in identical or similar form to any other German or foreign examination board.

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Summary

The work presented in the thesis was carried with the aim to understand how Widerborst (Wdb) regulate planar cell polarity in *Drosophila* wing. In search of proteins interacting with Wdb I carried a Yeast Two Hybrid screen and identified a protein, bitesize, with tandem C2 domains in its C terminus interacting with Wdb. Wdb also interacts with btsz genetically and removal of one copy each of Wdb and btsz enhances the truncated hair phenotype observed in Wdb EMS mutants and btsz P element insertion mutants.

There are at least three predicted isoforms of bitesize and loss of the btsz-II isoform is lethal. Clonal analysis of a btsz mutant, $btsz^{J5\text{-}2}$, which removes the btsz II isoform resulted in truncated wing hair outgrowth. On the other hand over expression of a myc-btsz-II construct resulted in hair duplication phenotype. However, over expression of the GFP-CT is sufficient to give wing hair duplication phenotype and this hair duplication phenotype is stronger than that caused by myc-btsz-II over expression.

The Myc tagged btsz-II protein shows apical localization. Though most of the protein is confined to cytoplasm, btsz-II also marks the plasma membrane. The GFP-CT construct marks the plasma membrane strongly and is enriched in the apical region. The over expression of CT domain is sufficient to give hair duplication phenotype and the strong difference observed in the localization pattern of full length btsz-II protein and GFP-CT together suggest that regulation of membrane localization of btsz through its CT region is important to regulate hair morphogenesis.

As the loss of function (truncated wing hair) and gain of function (hair duplication) both affect the process of hair morphogenesis, it can be said that btsz is a positive regulator of hair morphogenesis. Since no defect in cortical polarization of Fmi was observed in cells lacking btsz-II, btsz is required for establishment of cortical domains. However with the present study it remains unknown how exactly the C2 domains might regulate hair morphogenesis and whether Wdb targets btsz for dephophorylation to PP2A catalytic subunit.

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Chapter I: Introduction

1.1 Cell Polarity

Most of the cell types exhibit polarity at some stage. Unicellular organisms like yeast show polarization during budding (Casamayor A et al., 2002). Migrating cells show polarization of the cytoskeletal machinery at the leading age lamella (Vincente-Manzanares M et al). The cells of immune systems, T lymphocytes and antigen representing cells (APC) interact with each other by polarizing the adhesion molecules ICAM-3 and ICAM-1 (Montoya M et al., 2002). Tissues derived from epithelial cells show polarized organization vital for their proper functioning. Few examples of functions dependent on polarized tissue organization include secretion by epithelial glands like salivary glands, uptake of nutrients by inner lining of intestine or the coordinated movements of epithelial sheets during morphogenesis (Muller and Bossinger, 2003).

1.2 Apical-Basal Polarity

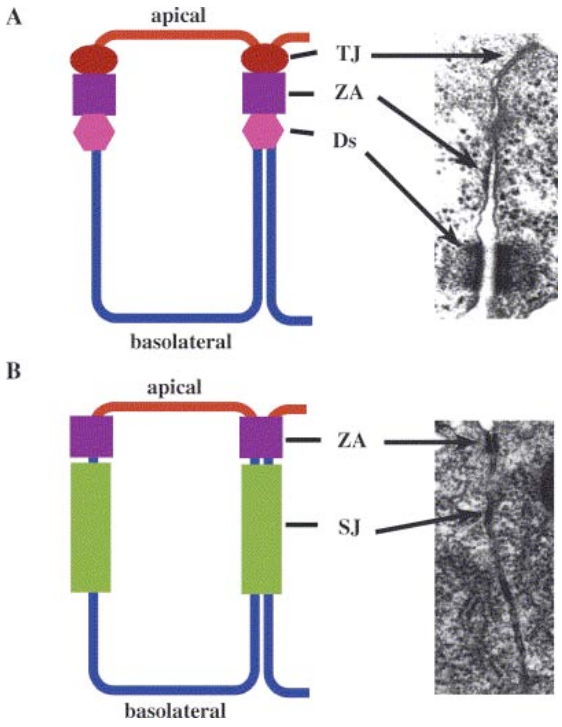


Fig. 1: Cell surface polarity of vertebrate and non-vertebrate epithelia. The right hand panel shows transmission electron micrograph of vertebrate (amphibian) embryo and invertebrate (*Drosophila*) embryo. (A) The vertebrate junctional complexes consist of tight junctions (TJ), zonula adherens (ZA) and desmosomes (Ds). (B) The invertebrate epithelial junctional complexes consist of apical ZA and lateral septate junctions (SJ) (From Muller and Bossinger 2003).

Apical-basal polarity is a characteristic feature of epithelia cells and tissues derived from epithelia (Muller H., 2000). Specialized cell junctions divide the plasma membrane into apical and basal sub-domains. The apical surface is in contact with the surrounding environment while the basal side is in contact with extracellular matrix composing the basal lamina. The tight junctions serve as diffusion barrier between the apical and basolateral surfaces. The adherens junctions provide the adhesion between neighbouring epithelial cells. Loss of this adhesion can lead to unregulated migration leading in the end to cancer. Another type of adhesive junction is the desmosomes, which are present only in vertebrates. The organization of these junctions differs slightly from vertebrates to invertebrates. In *Drosophila* the tight junctions are absent and their function is taken over by septate junctions (Tepas U et al., 2001). Not only proteins but lipids are also asymmetrically distributed in the apical and basolateral cell surfaces. For example glycosphingolipids (GSLs) are highly enriched in the outer leaflet of apical plasma membrane of polarised epithelial cells (Hoekstra D et al., 2003).

The apical surface of various epithelial tissues is modified according to the function of the tissue. For example the apical membrane of the gut cell lining is thrown into microvilli to increase the surface area or the apical surface of cells of respiratory tract give rise to cilia (Nelson W., 2003)

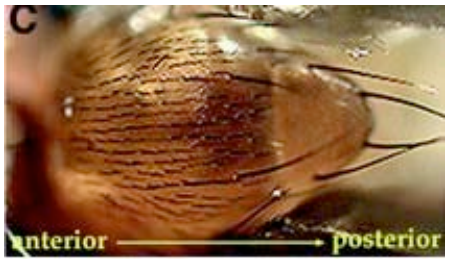
Thus, epithelial cells organize their plasma membrane in apical and basal domains perpendicular to the axis of plane and further modify the apical surface as per functional needs.

1.3 Planar Cell Polarity

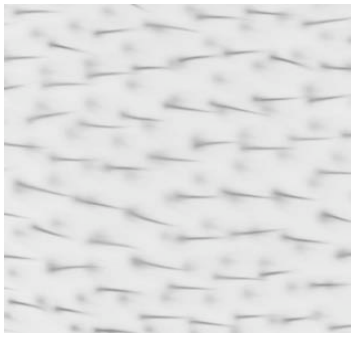
In some tissues in addition to the apical-basal axis of polarity, additional axis of polarity is required for proper functioning. The cilia of respiratory tract are oriented in the same direction. This orientation is important for harmonized ciliary beating in the same direction (Uemura T et., al 2003). Another remarkable example of planar polarity is sensory hair cells (stereocilia) of inner ear of mammals. The staircase like arrangement of sterocilia is important for the mechano-sensory transduction and thus for hearing ability (Lewis J. et al., 2002). In all these cases the outgrowths from the

apical surface of each cell are arranged and oriented in the plane of the epithelium. This arrangement results in a tissue polarized in the plane.

a) The Notum of *Drosophila*

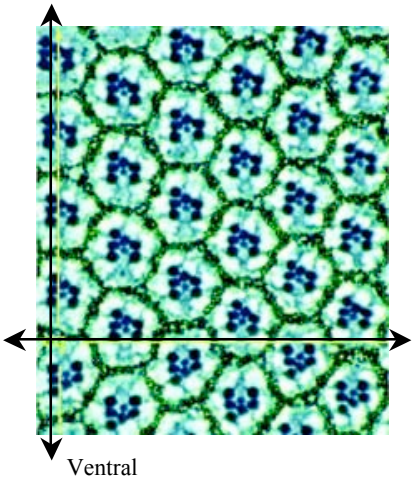


b) *Drosophila* wing Hair

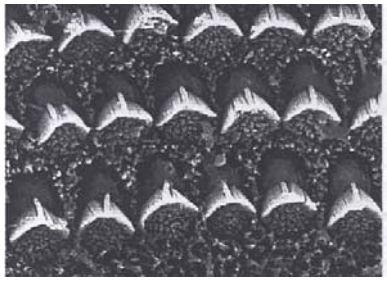


c) Drosophila eye

Dorsal



d) Inner hair cells of organ of Corti



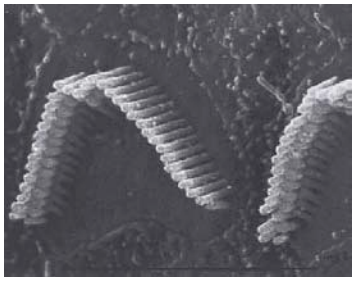


Fig. 2: Various polarized tissues: a) *Drosophila* thorax shows posteriorly oriented sensory bristles and hairs. b) *Drosophila* wing region between L3-L4 veins/. c) The compound eye of *Drosophila*. Each ommatidium consists of photoreceptor cells arranged in a mirror image manner across the dorsal-ventral axis (from Mlodzik 1999). d) Scanning electron micrographs of the surface of the mouse organ of Corti (From Self 1998).

Tissue polarity is also evident in the cuticle secreted by ectodermal epithelia of insects. The cuticular surface of *Drosophila* is decorated with hairs and sensory bristles. Each wing cell of *Drosophila* produces a single hair pointed distally (Eaton S. 1997). The skin of higher vertebrates e.g. the feathers of birds or the scales in fish also shows polarized arrangement.

1.4 Planar Polarity in *Drosophila*

The cuticle of *Drosophila* is decorated with hairs and bristles. In most body regions these structures are aligned in parallel and point in common direction. That gives the tissue a vectorial pattern resulting in tissue polarity. Three main types of cuticular structures have been analysed in *Drosophila* with respect to tissue polarity. These include hairs (trichomes), sensory bristles and the eye ommatidia (Alder P. 1992).

Each hair is produced from the apical surface of a single epidermal cell. The sensory bristle organ is made of five cells (socket, shaft, sheath, neuron and glial cells) (Gho and Schweiguth 1999). The polarity in eye is much more complicated and is reflected by the mirror image arrangement of ommatidia of opposite chiral forms across the dorso-ventral midline (Adler 1992). In case of sensory bristles and eye ommatidia the regulation of planar polarity also involves differentiation of the sensory organ cells and photoreceptor cells respectively. Although tissue polarity is evident in various body regions (notum, abdomen, eye, legs, wings etc) the wing had been the favourite tissue for analysis of tissue polarity. Wing is relatively homogenous and simple tissue. The morphogenesis of wing is extensively studied and being a flat tissue it is easier for microscopic analysis.

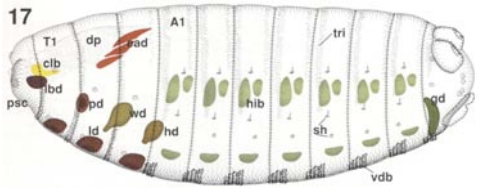
1.5 Wing Development

The adult wing and other epidermal structures have their origin in specific sacs of cells called “imaginal discs”. Imaginal discs develop from invaginations in the ectoderm during late embryonic/ early larval stage and grow inside the body cavity of larva. Each imaginal disc is composed of a single layer of columnar epithelium (disc proper) and a thin layer of squamous cells called peripodial membrane. Cells of the

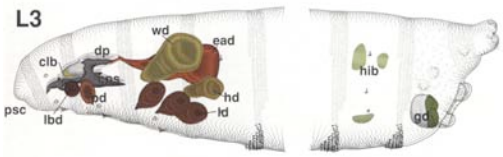
disc epithelium have an apical basal polarity like that of other epidermal epithelia of insects (Ashburner Vol. 2d). While the imaginal discs grow and mature on the inside of the larva they remain connected to the larval epidermis by their peripodial stalk.

Three different secreted proteins namely Wingless (Wg, belong to Wnt family) Hedgehog (Hh) and Decapentapleigic (Dpp, member of BMP, bone morphogenic protein, family) play a major role in patterning the wing imaginal disc (Tabata T et al., 2004). At late third instar stage Wg is expressed in a stripe defining the dorso-ventral boundary. Wg can be found 25 cells diameter away from producing cells. Frizzled and Dfrizzled-2 act as receptors for Wg (Cadigan K 1998).

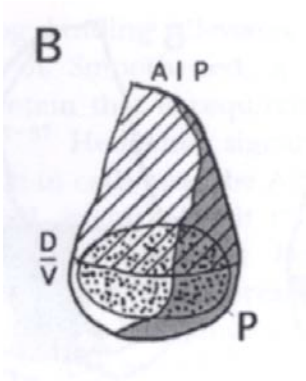
a) Stage 17 embryo



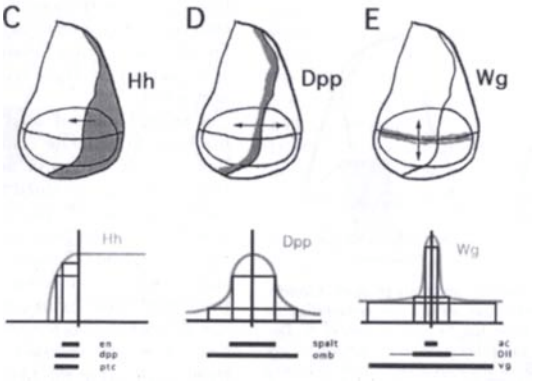
L3 larva



b) 3rd instar Wing imaginal disc



Morphogen expression in wing disc



c) Adult wing

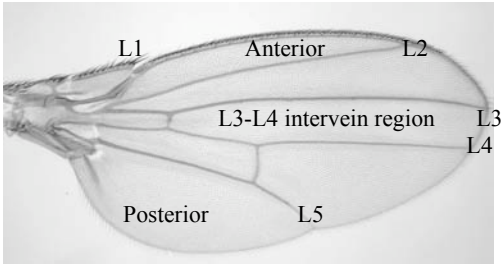


Fig3: Development of wing: a) Schematic presentation of imaginal discs at stage-17 embryo and 3rd instar larva. (ld= leg disc, wd= wing disc, hd= halter disc, gd= genital disc, lbd= labial disc, ead= eye antennal disc, hib= abdominal histoblasts, dp= dorsal pouch) (From Atlas of *Drosophila* Development, CSHL press). b) The third instar larval wing imaginal disc depicting pattern of expression of the three different morphogens (A- anterior, P- posterior, D- Dorsal, V- ventral) (From Strigini &. Cohen 1999) c) Adult wing with anterior and posterior compartments separated by L3-L4 inter vein region.

Only the cells of the posterior compartment express Hh. The adjacent anterior cells, over 10 cells diameter, receive the secreted Hh and this induces expression of Dpp. Dpp in turn acts as a long-range morphogen. Patched and Thick-vein proteins act as receptors for Hh and Dpp respectively (Strigini M et al., 1999). Hh, Dpp and Wg producing cells act as organizers and define the anterior-posterior and dorso-ventral axis respectively. Upon secretion from the producing cells these morphogens form a concentration gradient. The receiving cells response to the concentration gradient by differential level of gene expression of the target genes (Freeman M. et al., 2002). Shortly after pupariation the imaginal discs evaginate to assume their position at the surface of the prepupal animal and undergo extension. During the process of evagination and extension the wing accumulates fluid between the bi-layer of cells forming a sac (Brown N et al., 2002).

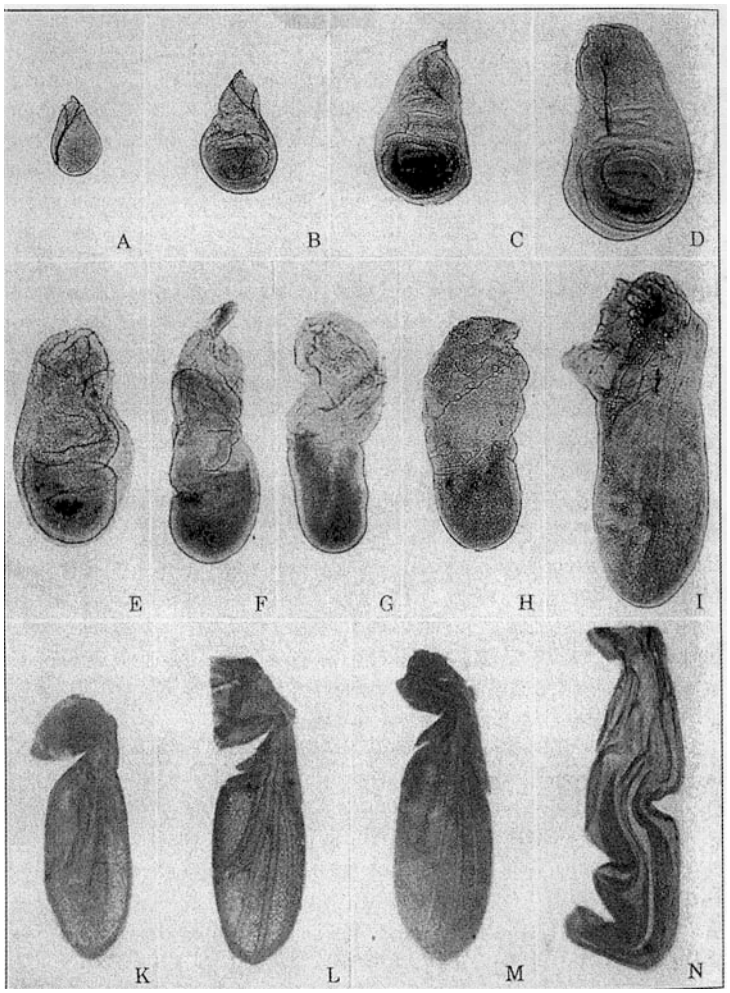


Fig.4: Various stages in the development of wing. A-D successive stages of 3rd instar wing disc. E-I: Successive stages of transformation of the wing disc during puparium formation and pupation. K: wing of 18Hr old pupa, L: wing of 28Hr old pupa. M: wing of 38Hr old pupa, N: Wing of 48Hr old pupa.(From Biology of *Drosophila*: Hafner Publishing Company).

The area forming the thoracic notum is marked off from the wing blade by a hinge. This hinge deepens during the stages of pupal formation. Approximately 48Hr after puparium formation (apf) the flattened wing undergoes folding that is retained till the emergence of imago. Within one hour of emergence the wing is flattened with pressure formed by the fluid stored between the two layers of epithelia. The adult wing has about 30,000 cells and remarkably each one of them having a single, distally oriented hair outgrowth (Mitchell H et al., 1983).

1.6 Cell Biology of Wing Hair Formation: Cytoskeletal Rearrangements and Vesicular Traffic

Each wing epithelial cell develops a hair outgrowth in the distal region. The process of hair formation initiates approximately 30Hr after puparium formation (apf). Hair extension is complete approximately by 35 Hr apf (Fristrom D et al., 1983). This is followed by deposition of double layer of cuticulin on cell surface. After hair formation the cell undergoes transition in its shape from columnar to flat (Mitchell H et al., 1983).

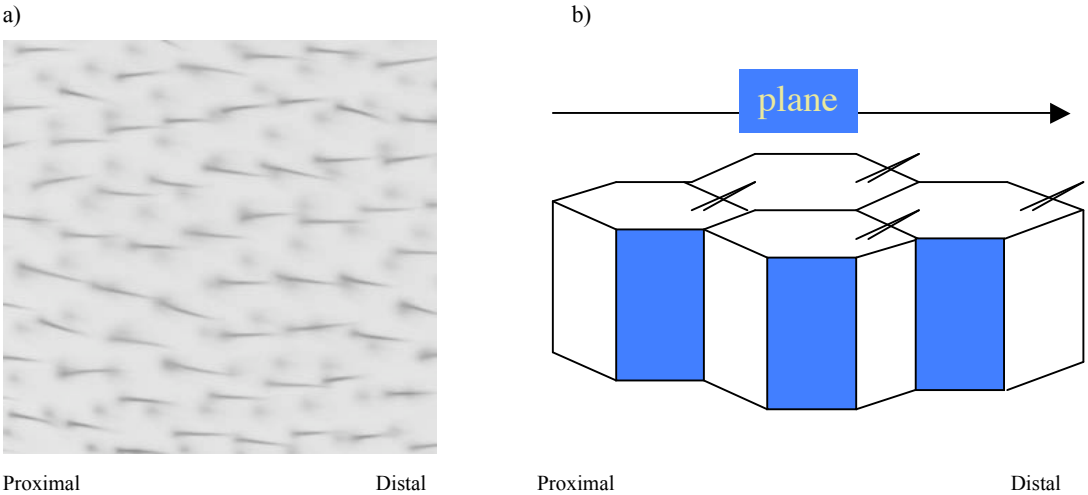
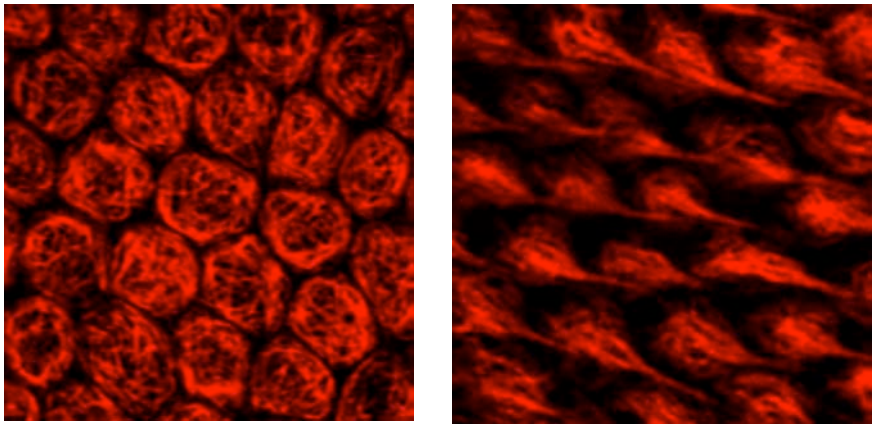


Fig 5: a) Light microscopic image of dorsal surface of wing. b) The cartoon on left shows diagrammatic presentation of wing epithelial cell. Each wing cell forms a single hair outgrowth in the distal region.

Cytoskeletal Rearrangements: The microtubule cytoskeleton plays a major role in hair formation. One of the most striking features of wing epithelial cells is the presence of a microtubule web at the level of apical junctions (Eaton S. 1996). The microtubule web is vital for hair formation and undergoes dramatic modification

during hair formation. At initiation of hair formation the apical microtubules undergo elongation and start accumulating distally. The elongated microtubules fill the extending hair outgrowth.



a) 26 Hr apf: Ace tubulin b) 31 Hr apf Ace tubulin

Fig.6: The microtubule web of the wing epithelium undergoes structural reorganization and fills the hair outgrowth. a) Microtubules at 26 Hr apf stage. b) A wing with microtubules filling the extending hair at 31Hr apf. Microtubules are marked with Mouse anti Ace tubulin antibody. Proximal is left and distal is right.

In addition to microtubules, actin also plays a vital role in hair morphogenesis. Before hair formation actin in the wing cells is organized along the cell cortex in apical region. As first sign of hair formation filamentous actin accumulates at the distal vertex of each cell forming a prehair (Wong et al., 1993). This filamentous actin fills the extending hair. Actin and microtubules fill the extending hair almost simultaneously and hence it is difficult to point out which event takes place first.

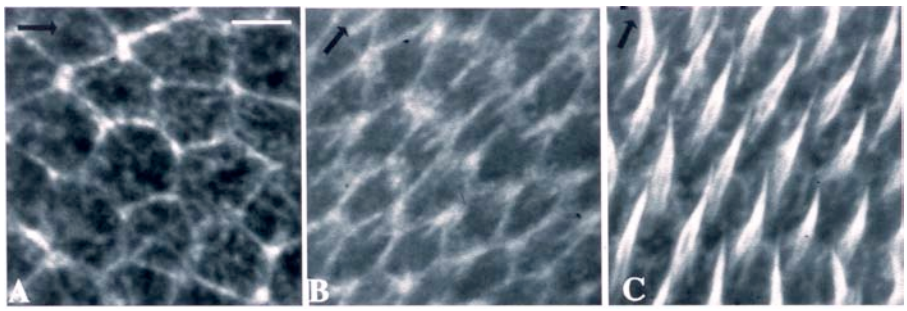


Fig.7: Actin cytoskeleton reorganization during hair formation (30-35 Hr apf, left to right). Actin is marked with phalloidin. The middle image shows accumulation of actin in the distal region prior to hair formation. The image on right shows actin filaments filling the hair outgrowth (From Eaton S. 1996).

Treating the wing with cytochalasin D (inhibitor of actin polymerization) or vinblastine (microtubule depolymerising drug) shows range of effects on hair morphogenesis depending on the time and concentration of drug application. The

effect varies from total inhibition of prehair formation, delay in elongation of prehair to multiple hair phenotype (Turner and Adler 1998).

Vesicular Trafficking in Hair Morphogenesis: Though there is not enough information available about the nature of vesicular trafficking machinery involved in hair formation movement of materials in and out the developing hair has been documented (Mitchell et al., 1983). Pupal wings isolated at time of hair outgrowth can internalize fluorescent dye fastDI (a dye which integrates in outer leaflet of plasma membrane) and migrated in discrete dots into the growing hair (Hannus M. unpublished data). Blocking function of dynamin at the time of hair formation using temperature sensitive allele of *shi* results in stunted hair outgrowths (A. Classen, unpublished data). These data indicate involvement of membrane transport in hair morphogenesis.

Products of furry (*fry*) and tricornered (*trc*) genes are known to be involved in maintaining the integrity epidermal cell extensions including hair outgrowths (Cong et al., 2001; Geng et al., 2000). Mutations in *trc* and *fry* results in dramatic multiple wing hair phenotype and split hair phenotype. Recent work shows that Trc, a Ser/Thr protein kinase belonging to NDR kinase family, and Furry (Fry) are found to the cell periphery prior to hair formation and later in the growing hairs in punctuate pattern (He Y et al., 2004). This is consistent with the earlier observations that membrane transport is required for hair formation. Furry (Fry) and Trc physically interact with each other and their localization is interdependent indicating they are involved in a common signalling pathway. In addition phosphorylation site mutants of Trc show reduction in accumulation in the growing hair indicating phosphorylation activity of Trc is important for its subcellular location.

The small GTPase Rho family member cdc42 and Rac1 have been shown to regulate hair formation. Cdc42 regulates actin polymerization and cells lacking of Cdc42 GTPase activity often fail to generate hair outgrowths. Cdc42 activity is required in general for polarized membrane outgrowths like filopodium of fibroblasts and polarized budding in *Saccharomyces cerevisiae*. On other hand cells expressing dominant negative form of Rac1 fail to restrict hair outgrowth at single location

resulting in to multiple wing hairs that are structurally normal. Also the apical microtubule web is disorganized in these cells (Eaton S. 1996). The multiple wing hair phenotype caused by destabilization of microtubules by vinblastine treatment (Turner and Alder 1998) is in line with microtubule disorganization observed in cells expressing dominant negative Cdc42. Altogether this data suggests that both actin and microtubule cytoskeleton is required for hair formation and regulation of microtubules is important to form single hair outgrowth.

Loss of *Drosophila* Rho associated kinase (Drok) activity results in hair duplication phenotype. Drok was shown to regulate actin cytoskeleton by regulating phosphorylation level of non muscle myosin regulatory light chain (Winter et al., 2001). Drok also interacts genetically with Zipper (Zip, *Drosophila* myosin II) and Crinkled (Ck, *Drosophila* myosin VIIA). Intriguingly, mouse myosin VIIA (shaker) is implicated in Usher Syndrome 1B (Self et al., 1998). This indicates that some aspects of hair formation machinery of *Drosophila* and inner ear of mammals are conserved. Ste20 kinase homologue, Misshapen (Msn), is also required for hair formation. Wing cells homozygous mutant for *misshapen* fail to form hair. Msn also cause planar polarity defects in the eye (Paricio et al., 1999).

Taking into consideration the present cell biological evidence regulation of polarized reorganization the actin-microtubule cytoskeleton and transport of material into the growing hair seems to be critical for proper hair outgrowth.

1.7 Regulation of Wing Hair Polarity: Three Tiers and Non-autonomy

Three Tiers: The present data indicate that planar cell polarity is tightly regulated and complex phenomenon. The cellular machinery involved in establishment of wing hair polarity can be divided in three tiers (Tree et al., 2002a). The first tire involving the Fat/ Dachsous/ Four-jointed cassette, the second tire compromising the core members forming cortical domain and the third tire made up of the players acting downstream Fz signalling. Failure at any of these three stages can result in abnormality in planar polarity.

Before initiation of hair formation the site of hair formation is marked by apical accumulation of actin in the distal region of the cell. The site of prehair formation is regulated by Frizzled (Fz). Hair forms in the centre of the apical surface in absence of Fz activity (Wong and Adler 1993). Frizzled is a seven pass trans-membrane protein and act as a receptor for canonical Wingless pathway redundantly with DFz2 (Muller et al., 1999). Though Fz is proposed to act as receptor in planar cell polarity (PCP) pathway there is no proof for involvement of Wnts in PCP pathway. Dishevelled separates the canonical Wg pathway from the PCP pathway. The DEP domain of Dishevelled acts specifically in the PCP pathway while its DIX domain is required for canonical Wg signalling pathway (Axelrod et al., 1998).

Several other genes are thought to act together with *fz* to regulate planar cell polarity. A 'core group' of genes regulating tissue polarity has been identified (Gubb D. 1993). In addition to *frizzled* (*fz*) these include *dishevelled* (*dsh*), prickle (*pk*), *Van-Gogh/ strabismus* (*Vang/ stbm*) and *flamingo/ starry night* (*fmi/ stan*) and *diego* (*dgo*). Lack of any of these core components lead to polarity defects in all three tissues (wing, sensory bristles and ommatidia) analysed in *Drosophila*. The second group of genes alters tissue polarity in tissue specific manner. This group includes *inturned* (*in*), *fuzzy* (*fy*) and *multiple wing hair* (*mwh*). Epistatic analysis indicated that the second group genes act downstream of the core group of genes (Wong and Adler 1993).

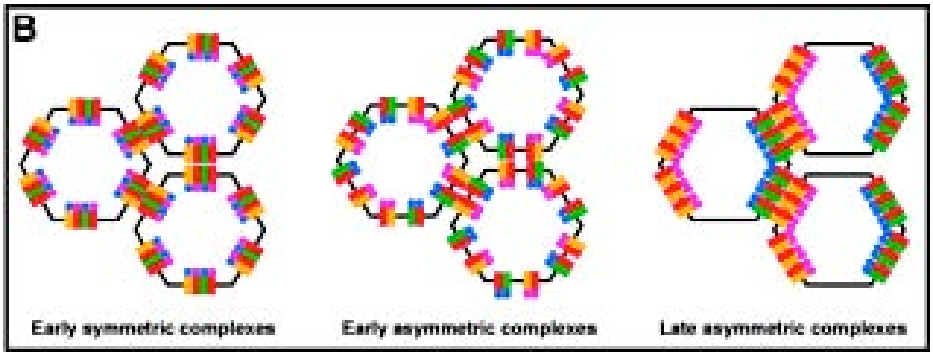


Fig.8: Models for asymmetric proximal-distal localization of core protein in planar cell polarity: Proteins represented in the cartoon here are Fmi (red), Fz (green), Dsh (blue circles), Stbm (yellow) and Pk (purple). During early stages the complexes involving these proteins are proposed to be distributed apical, symmetrically or randomly oriented relative to proximal-distal axis. Ultimately the pattern evolves to attain perfect asymmetry (From Bastock et al., 2003).

One of the most important feature of members of the core group is they acquire asymmetric distribution along the proximal-distal axis (Eaton S. 2003; Strutt D.

2003). Approximately 6 to 10 hr prior to hair formation these proteins polarize their distribution at the level of apical junctions and forms distinct proximal or distal domains. Fz (Strutt D. 2001) and Dsh (Axelrod J. 2001) become asymmetrically localized to the distal vertex of the cell, marking the site of subsequent hair formation. Fmi (Usui T. et al., 1999) is an atypical cadherin protein with seven pass trans-membrane domains and is thought to be involved in homophilic adhesive interactions. This adhesive interaction is important for polarization and in absence of Fmi the two other trans-membrane proteins Fz and Stbm (also known as Vang, Taylor et al., 1998) fail to recruit at the junctional level (Bastock et al., 2003). Dgo is an ankyrin repeat protein and is thought to localize both proximally and distally (Feiguin F. et al., 2001). Stbm and Pk are found at the proximal cell edges (Bastock R et al 2003; Tree et al., 2002b). Lack of activity of any of these core proteins result in failure of asymmetric distribution of the others. This indicates that these proteins act together in multi-protein complexes.

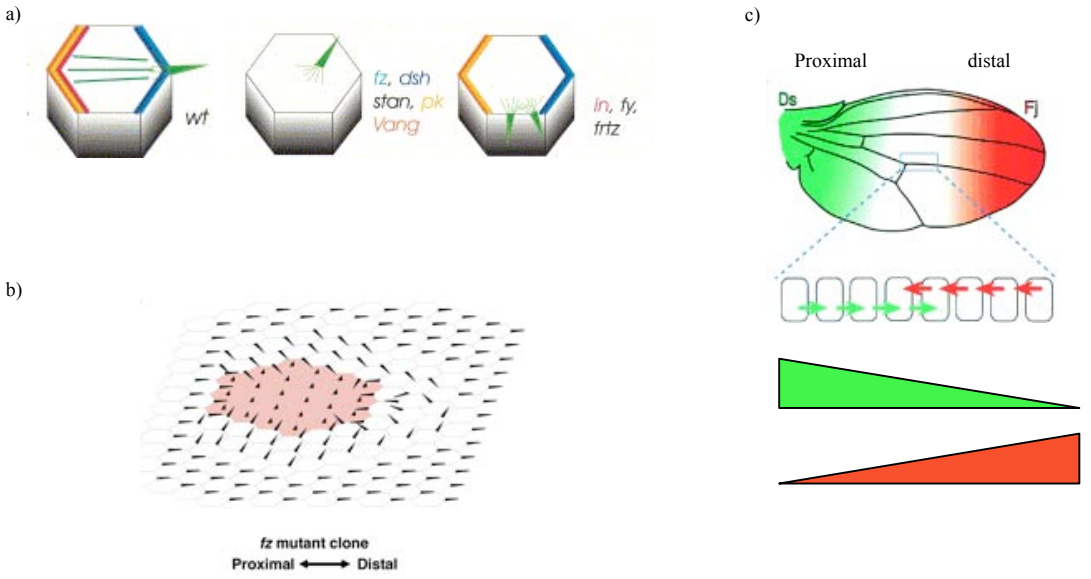


Fig. 9: a) In wild type cells the wing hair id formed in the distal region of the cell. However in the lack of activity of any of the core group members results in hair formation in centre of the cell (Modified from Adler et al., 2004). b) Non autonomy in *fz* mutant clone: while cells lacking Frizzled form wing hair in centre of the cell, the tissue surrounding the clone orients the wing hairs towards the clonal cells (Modified from D. Strutt 2003). c) Model showing gradient expression of Dachsous and Four-jointed in pupal wing (Modified from Uemura and Shimada 2003).

Non Autonomy in Planar Polarity: In addition to its core function in regulating hair site formation Fz exhibits long range non autonomous effects on hair polarization. In

clones of cells lacking fz activity the hairs are formed in the centre of cell and are directed away from the clone giving a swirling pattern. At the same time cells with endogenous fz activity surrounding the clone orient their hair towards the clone resulting into non autonomy (Gubb and Garcia-Bellido 1982; Vinson and Adler 1987). Clones of another tissue polarity *Van Gogh* (Vang, also known as *strabismus*) display complementary domineering non autonomy. Wild type cells surrounding clone of cells lacking Vang activity point their hairs away from the clone (Taylor et al., 1998). Similarly *pk* clones are also known to show non autonomous phenotype. Considering these phenotypes and the genetic interaction between these loci, it has been proposed that these proteins act together to regulate long range polarity signal. The presence of this non-autonomous phenotype indicates the possibility of propagation of Fz mediated PCP signal across the tissue.

Two more atypical cadherins Dachsous (Ds) and Fat (Ft), and a type II trans-membrane named Four-jointed (Fj) show non autonomous defects in planar polarity and are proposed to provide global directional cues for propagation of Fz signalling (Ma D. et al., 2003). The Fat/Dachsous/Four-jointed cassette acts upstream of Fz mediated planar polarization. Ft and Ds localize apical to adherence junctions and do not show any asymmetric distribution. In contrast Fz is observed at the level of adherens junctions and polarize efficiently but along wrong axis in *fat* or *dachsous* mutant tissue (Ma D et al., 2003). In contrast, apical localization of Ft and Ds is not affected in *fz* mutant tissue. Ft and Ds have been shown to be involved in heterophilic adhesion interactions instead of homophilic adhesion (Matakatsu and Blair 2004). While Ft is uniformly expressed in the wing, Ds show high expression level in the proximal region and Four-Jointed shows high expression in the distal region of the wing. This can lead to difference in the type of adhesions maintained in the proximal and distal regions of the wing. However, how this difference is transmitted to Fz signalling is not clear.

A non-core (tissue specific) group of downstream effectors is involved in regulation of both wing hair morphogenesis and orientation. This non-core group includes *inturned* (*in*), *multiple wing hair* (*mwh*), and *fuzzy* (*fy*). Inturned is a putative trans-membrane protein and in wing cells it forms a proximal cortical domain. In absence of

Fuzzy (Fy) and Fritz (Ftrz), Inturned fails to polarize indicating that Fy and Ftrz are essential for polarization of Inturned. Though Inturned is polarized in cells lacking the core proteins like Fz, in cells over expressing Fz along anterior-posterior compartment boundary, the axis of Inturned orientation is altered from proximo-distal to anterior-posterior (Adler P et al., 2004).

1.8 Widerborst In Regulation of Wing Hair Outgrowth and Polarity

One important finding in understanding of cell biology of planar cell polarity in wing was discovery of Widerborst (Wdb), a *Drosophila* Protein Phosphatase 2A (PP2A) regulatory subunit (Hannus M et al., 2002). Discovery of Wdb was important for various reasons. Wdb is the most upstream protein known to be polarized and shows dynamic pattern of polarized localization. At 5Hr apf Wdb shows proximal accumulation but shows distal polarization as early as 18 Hr apf. However, Wdb do not co-localize with other cortical domains instead it localizes on the distal region of the apical microtubule web. This indicates that prior to establishment of core cortical domains clues are available for establishment of planar polarity (at least in the pupal wing epithelium).

Further more Wdb is required for both proper hair morphogenesis and establishment of cortical domains. While over-expression of full length Wdb results in hair duplication phenotype, over-expression of an N-terminally truncated dominant negative form not only causes various abnormalities in hair morphogenesis (truncated /stunted wing hair, bald patches) but also results in abnormal hair polarity. This suggests loss of regulation at various stages in the establishment and maintenance of polarity.

The regulatory subunits of PP2A direct specific substrates to the catalytic subunit of PP2A in various signalling pathways (Sontag E 2000; Janssens and Goris 2001). Though the microtubule web as such does not show any structural polarity, the distal microtubule localization of Wdb and its role as regulatory subunit suggests possibility that the distal region of the web is functionally polarized. The dominant negative construct was designed in order to bind putative substrates but failing to target them to

the catalytic subunit (Ito A et al., 2000). This raises a question what substrates does Wdb target to microtubule star (Mts), the PP2A catalytic subunit of *Drosophila* PP2A in order to regulate planar polarity in wing.

Since the loss of function of the core group of proteins do not affect hair morphogenesis, it is highly possible that Wdb interacts with different sets of proteins to regulate planar polarity and hair morphogenesis. The microtubule web is also disorganised in cells expressing dominant negative Wdb. This can result in abnormal hair morphology since wing hair is filled with microtubules. It is also possible that the disorganized microtubule web results in altered localization of some microtubule dependent proteins and that indirectly affects hair polarity and/or hair morphology. Thus expression of dominant negative Wdb can result in various possibilities ultimately affecting to both hair polarity and morphogenesis.

A genetic screen designed for suppressors of Wdb over expression resulted in intragenic mutations leaving the question of genetic interacting partners unanswered. Thus the genetic and protein-protein interactions in which Wdb might be involved to regulate different aspects of planar polarity remained a mystery.

1.9 Planar Polarity Components In Vertebrate Gastrulation

Many recent studies indicate that several components of Drosophila PCP pathway also operate in vertebrate embryos in regulation of convergent extension. During gastrulation the mesodermal cells elongates along an axis and narrow along the perpendicular axis (convergence) to provide an antero-posterior axis for extension. This process of convergent extension (CE) is important since it defines the axis of cell movements during gastrulation. Similar processes are involved in neurulation and organogenesis. (Keller R., 2002).

CE is considered as a manifestation of planar polarity since the cells become polarised in plane of gastrulation line. However CE in vertebrates is observed in both ectodermal and mesenchymal tissues. While in the wing planar polarity is manifested in epithelial tissue in form of apical hair outgrowth filled with actin and microtubules,

in CE the polarized extensions are protrusive in nature. The common feature is polarized actin rich cell surface extensions. Studies in *Xenopus* and zebrafish embryos indicate that many components of the PCP pathway are also involved in CE. The first connection was established with the discovery that mutant forms of Dsh that disrupt PCP pathway but not the canonical Wnt pathway also disrupt convergent extension (Heisenberg et al., 2000; Wallingford et al 2000). However it should be noted that in contrast to PCP in fly wing in which none of the Wnt genes have been implicated, Wnt-11 (*silberlick*) and Wnt-5a (*pipetail*) control CE in vertebrates (Heisenberg CP et al., 2000; Moon et al., 1993).

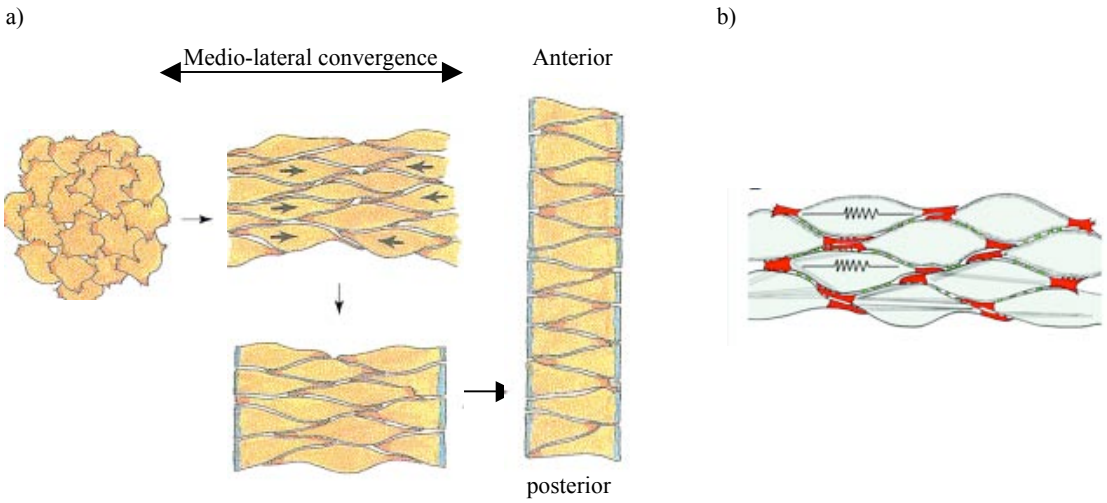


Fig. 10: Morphogenetic cell movements during convergent extension (CE): a) During mesodermal gastrulation the cells become polarised in a bipolar fashion to narrow down along one axis and elongate along the perpendicular axis (mediolateral convergence) and the tissue extends on the antero-posterior axis (Modified from Mlodzik M. 2002). b) Model showing how polarized protrusive activity (red regions) is thought to provide cell intercalation during CE (From R. Keller 2002).

So far most of the core group members involved in PCP in fly have been shown to act also in CE. Support for a conserved pathway come from involvement of *L-tap* and *trilobite* genes, the mouse and fish homolog of *strabismus* respectively, in regulation of convergent extension (Kibar et al., 2001; Jessen et al., 2002). Morpholino experiments suggest that the zebrafish homologue of Widerborst is also involved in CE though its precise function is not known (Hannus M et al., 2002).

There is also evidence that several downstream effectors involved in fly PCP are also required for CE. In zebrafish dominant negative Rho kinase disrupts mediolateral elongation, intercalation and also inhibits the extension (Marlow et al., 2002). A role for Cdc42 is suggested by the fact that a dominant negative form of Cdc42 can rescue inhibition of CE caused by over-expression of Frizzled-7 (Djiane et al., 2000).

1.10 Unresolved Questions In Planar Polarity

In past few years a lot of work is done in different laboratories in understanding planar polarity mainly using *Drosophila* wing as model system. This has resulted in discovery of machinery that seems to be conserved in different processes requiring polarization in plane. Though some components and their roles vary from tissue to tissue, a core group has been identified which is highly conserved from insects to vertebrates including mammals.

Establishment of PCP in wing can be subdivided in two major steps. First is establishment of cortical domains and the second step is polarized signalling by Fz culminating in wing hair morphogenesis. The first tier and the second tire (the core group) are required for proper orientation of wing hair but none of them is involved in hair morphogenesis. Further the involvement of the third tier indicates that even if the core cortical domains are established properly the mutations in some downstream components (eg. *inturned*) still can result in abnormal polarity. That leaves us with two open questions. A key question is identifying the early clues in establishment of cortical polarization of the core group members and Widerborst. The second is regulation of downstream components that are required for both polarity and hair morphogenesis.

Though Wnt family members have been shown to act as ligand in convergent extension no such ligand has so far been shown required for PCP in fruit fly. So how the Frizzled receptor is activated during PCP to signal via the DEP domain of Dishevelled in wing remains a mystery and an unknown factor X has been proposed. In this regard the early and dynamic polarization pattern of Wdb provokes interest. Wdb is the only component known to be localizing to microtubule cytoskeleton.

Being a regulatory subunit of PP2A it makes sense to argue that polarized localization of Wdb can manifest in polarised regulation of some of the known and unknown components of PCP pathway. Since expression of dominant negative Wdb results abnormalities in polarity and morphogenesis of wing hair, the components under regulation of Wdb might be involved both of these processes.

In my thesis work I sought to identify proteins interacting with Wdb and involved in regulation of PCP in wing. I will describe the role of one Wdb interactor, *bitesize,* in wing hair formation.

Chapter II: Materials and Methods

2.1 Yeast Two Hybrid Screen

The Matchmaker GAL4 Two Hybrid System-3 (CLONTECH) was used for the yeast two hybrid screen. The bait gene (wdb) was expressed as a fusion to the GAL4 DNA binding domain (DBD) in the yeast strain AH109. A pupal specific oligodT-cDNA library was constructed using poly-A RNA from 20-22 Hr apf (after puparium formation) stage pupae. The cDNA library was cloned in vector pGADT7 which allows expression of the library protein in fusion with GAL4 activation domain (AD). The system uses four reporter genes under control of heterologous GAL4 responsive upstream activating sequence (UAS) for identification of bait-library protein interaction. The reporter genes are HIS3, ADE2, MEL1 and lacZ.

cDNA Synthesis

Total RNA was extracted from approximately 100 pupae using TRIZOL based protocol. Poly-A mRNA was isolated using DynaBeads Oligo (dT)-25 (Dynal Biotech). cDNA was synthesized using Invitrogen Copy Kit (Catalogue No. L1311-03).

Modification of pGADT7 to accommodate cDNA library

In order to clone the cDNA library the prey vector pDADT7 (Clontech) was modified to incorporate a unique restriction site PspOMI. Following complimentary pair of Oligos was used to incorporate PspOMI site. The PspOMI site is highlighted in bold. The oligos were designed so that after annealing the 5' overhangs corresponding to restriction enzymes ClaI and XhoI (Marked by underline) will be generated.

GADada1: 5' <u>CGAT</u>CTAGA**GGGCCC**GGATCC 3'
GADada2 5' <u>TCGAG</u>GATCC**GGGCCC**TCTAGAT 3'

The annealed oligo was ligated with ClaI- XhoI (New England BioLab) digested vector using standard molecular cloning technique. This modified vector was used for cloning the cDNA library.

Cloning of pupal cDNA library and amplification in E.Coli

The cDNA library was used without normalization. The library was digested with NotI and then applied to Sephacryl column (S-400, PROMEGA) for purification. The eluted cDNA was cloned in SmaI and PspOMI digested pGADT7. The cDNA library transformed in XL10-Gold ultra competent cells (Invitrogen) as per instructions in the manual and the transformed bacteria were plated on LB plates with appropriate antibiotic. For amplification of the of the library the colonies from all plates were pulled and inoculated in 500ml LB and the culture was grown for 5 Hrs at 37^0C. The plasmid DNA was isolated using midi-prep kit (Quagen).

Bait Construct

To facilitate cloning of wdb cDNA (EST LD02456) was mutagenized to incorporate restriction site NcoI at the site of start codon. The mutagenesis was carried out using QuiKChangeTM Site directed mutagenesis kit (Stratagene). This modified wdb cDNA was digested with NcoI and XhoI (region from start Codon to complete 3'UTR) and cloned between NcoI and SalI restriction sites in pGBKT7.

Yeast transformation

Yeast strain AH109 was transformed using the Two Hybrid System TRAFO protocol (Gietz lab, The yeast transformation homepage). Stock of strain transformed with wdb was maintained on selective plate and was used for transforming with the AD domain fused cDNA library.

Yeast culture and growth media

The yeast strain AH109 was maintained on standard YPDA growth medium.
AH109 {pGBKT [wdb]} was maintained on SD –Trp medium and was used to screen the library.

Verification of Protein expression of the Bait in yeast

After transforming the yeast strain AH109 with the bait construct the expression of bait protein was checked by doing western blot analysis. 10 µl of cells were vortex mixed to resuspend in 50 µl of 1X Lamelli Reduced buffer. The sample was boiled at 950C for 5 min and chilled immediately on ice. 10µl of the supernatant was loaded on

10% SDS-PAGE gel. Standard western blotting procedures were followed. The blot was probed with rabbit anti myc antibody (sc-789, Santacruz Lab.) at dilution 1:1000. Donkey anti rabbit HRP tagged secondary antibody was used for detection. The detection reaction was carried with ECL detection kit.

Suppression of Auto-activation of -His reporter by bait

The auto-activation of the –His3p reporter by the bait was suppressed by incorporating 2.5 mM, 3-Amino Trizol (Sigma) in the selective growth media while screening for interacting partners.

Screening of cDNA library and verification of protein-protein interaction

The cDNA library was screened against Wdb protein. Positive clones were selected on SD –Trp/-Leu/-His/-Ade plates at 30^0C.

To verify the protein-protein interaction, the yeast colony was grown on SD –Leu medium. To recover the plasmid DNA the yeast cells were lysed in presence of SET buffer and Lyticase. The lysate was used to extract plasmid DNA using Quiagen Miniprep plasmid DNA isolation kit.

AH109 {pGBKT7} was transformed with plasmid DNA of the putative interacting partner to rule of false positives. In a separate reaction the plasmid DNA was used to transform AH109 {pGBKT7 [wdb]}. The transformants were plated on SD –Trp/-Leu/-His/-Ade plates to cross verify the protein-protein interaction.

Identification of the cross verified interacting partner

After confirming the protein-protein interaction the insert cDNA of the confirmed interacting clone was PCR amplified with T7 and GAD primers and the PCR product was sequenced.

The DNA sequence was analysed for its homology by doing nucleotide-nucleotide blast search (http://flybase.net/blast/), followed by protein domain search by SMART (http://smart.embl-heidelberg.de/).

2.2 Fly Stocks

Following fly stocks were used during the project:

Stock Name	Genotype	Function	Source	Application
Ap-Gal4	yw; Ap-Gal4/Bcg	Apterus Gal4 driver	Andrea Brand	Expression of UAS construct
Hh-Gal4	yw, hsFlp; Hh-Gal4/ TM6Tb	Hedgehog Gal4 driver	Andrea Brand	Expression of UAS construct
Tb-Gal4	yw; Tb-Gal4/TM6Tb	Tubulin Gal4 driver	Andrea Brand	Expression of UAS construct
Double Balancer	yw; Sp/Cyo; Dr/TM6Tb	2^{nd} and 3^{rd} chromosome balancer stock	Eaton Lab	Balancer stock
3^{rd} Chromosome balancer	w^{1118};; TM6Tb/ TM3Sb	3^{rd} chromosome balancer	Eaton Lab	Balancer stock
BL-11743	ry^{506} P{PZ}$btsz^{10418}$	P element insertion mutant	Bloomington stock centre	Genetic interaction with Wdb
EP-3567	w^{1118}; P{EP}$btsz^{EP3567}$/TM6B, Tb^{1}	P element insertion mutant	Exelixis Inc	Genetic interaction with Wdb
FRT82 $btsz^{J5-2}$	w; P{EP}$btsz^{J5-2}$/TM6B Tb	P element insertion mutant	Julia Serano	Clonal analysis
pGUS{btszII-myc}	w: pGUS{btszII-myc}/Cyo	UAS construct	Julia Serano	Over expression of N-Myc tagged btsz-II
pGUS{GFP-CT}	w; pGUS{GFP-CT}	UAS construct	Julia Serano	Over expression of CT region of btsz
G-30: Mosaic 23	yw,hsflp; FRT82 hsCD2 <y+>	Heat shock induced flippase	Eaton Lab	Mosaic Analysis

2.3 Antibodies

Primary Antibody	Source	Working dilution
1. Rat anti Dcadherin	Oda	1:200
2. Mouse anti rat CD2 (MCA1548)	Serotec	1:2000
3. Mouse anti Myc (OP10)	Oncogene	1:200
4. Mouse anti αAce-tubulin	Sigma	1:200
5. Rat anti α tyrosinated-tubulin	Serotec	1:200
6. Rabbit anti Flamingo	Eaton Lab	1:100
7. Rabbit anti Wdb	Eaton Lab	1:200

Secondary Antibody	Fluorophore	Source	Working dilution
1. Donkey anti rat	Cy5	Jackson Labs	1:1000
2. Donkey anti rat	Alexa 488	Jackson Labs	1:1000
3. Donkey anti mouse	Alexa 488	Jackson Labs	1:1000
4. Donkey anti mouse	Cy3	Jackson Labs	1:1000
5. Donkey anti rabbit	Cy5	Jackson Labs	1:1000
6. Donkey anti rabbit	Cy3	Jackson Labs	1:1000

2.4 Genetic Interaction Between wdb and btsz

Virgin w; FRT82 wdb^{14} / TM6Tb (X) male w; $btsz^{EP3567}$ / TM6Tb

The F1 non-tubby adults were collected.

2.5 Mosaic Analysis

Mitotic clones of $btsz^{J5\text{-}2}$ were generated using FLP Recombinase as described by (*Golic et al Science, 1991; Xu and Harrison, Methods in Cell Bio Vol44*). The technique is based on heat shock induced expression of FLP Recombinase to catalyze recombination between the FRT sites inserted close to the centromere.

Cross strategy for generation of clones:

Virgin yw, hsFLP ; FRT82 hsCD2 <y+> (X) Male w; $btsz^{J5-2}$ / TM6Tb

The crossed flies were transferred to fresh vial on alternate days. Heat shock was given at 37.5^{0}C for 1:30Hr when the Tubby larvae started crawling and the non tubby larvae reached the 3rd instar stage. After heat shock the vial was returned to 25^{0}C. Non tubby pupae were collected and staged as 0-hr apf (after puparium formation) when they were still white.

A second 1-Hr heat shock at 37^{0}C was given to induce the heat shock dependent expression of CD2. After recovery for 1 Hr at 25^{0}C the pupae were dissected in PBS and the formaldehyde fixation protocol was followed.

2.6 Formaldehyde Fixation Protocol For Pupal Wings

This protocol is useful to fix filamentous actin. Pupae were aged to 24-32 Hr at 25^{0}C apf as per requirement of the experiment.

1. The Pupae were dissected in a drop of PBS on the lid of a Petri plate (FALCON) to remove the pupal case using fine P5 forceps.
2. The head end and abdomen end were punctured from the dorsal side and the visceral material was removed. Care was taken to keep the pupal wings undamaged and intact to the ectoderm.
3. The cuticular sack covering the wing was opened from the proximal side as much as possible to allow the entry of the fixative and the tissue was transferred to 4% PFA made in PBS.
4. After 5 min in PFA, the wings were made free from the cuticular sack, this was done in PFA and the individual wings were collected in PFA in a 24 well plate and fixed for 15 min.
5. At this stage the wings are fixed and after one wash in PBT (1X PBS with 0.1% Triton X 100) can be used to follow standard Immunostaining protocol.

2.7 Methanol Fixation Protocol For Pupal Wings

This protocol was followed to fix the microtubules.

1. The pupal wings were dissected as described above and after opening the cuticular sack covering the wing from the proximal side the tissue was transferred to 100% methanol pre-chilled at 4^0C and kept on ice in a Petri plate for 3-5 minutes. Care was taken not the stretch the tissue since this can result in damage to the microtubule web.
2. After 5 min on ice, the plate was moved to -20^0C for 15 min.
3. The tissue was re-hydrated with 5 min each on ice with grades 90% and 50% methanol: water.
4. All the wings (still in the wing sack) were collected in 50% methanol and stored on ice.
5. The cuticular sack was removed to make the wings free, this was done in 50% methanol at room temperature and then the individual wings were transferred to PBT and standard protocol for Immunostaining was followed.

2.8 Immunostaining of The Pupal Wings

Unless mentioned all steps were carried out at room temperature.

1. The methanol fixed or PFA fixed pupal wings were rinsed once in PBT and then permeablised for 15 min in 500µl PBT (PBS with 0.1% Triton X 100) on a shaker platform.
2. The tissue was blocked in PBT with 5% FCS (fetal calf serum, GIBCO BRL) for 15 min.
3. After blocking the PBT was replaced with 300µl of PBT with 5% FCS and appropriate primary antibody was used with the working dilution. The tissue was incubated overnight at 4^0C on a rocking platform.
4. The primary antibody as removed and the tissue was rinsed once with PBT and followed by 3 washes with PBT for 15 min each.

5. Prior to staining with the fluorescent labelled secondary antibody the tissue was blocked with PBT with 5% FCS.
6. The secondary antibody was diluted 1:1000 in PBT with 5% FCS and incubated for 1Hr 30min on a rocking platform.
7. The tissue was rinsed with PBT washed as after the primary antibody incubation.
8. The immunostained tissue was mounted in ProLong anti-fade mounting medium (Molecular Probes Inc. Eugene USA).

2.9 Preparation of adult wings for dark and bright field transmission microscopy

Adult flies were collected in an eppendorf tube in fixed in isopropanol at room temperature. The fixed flies were collected in a Petri plate containing isopropanol and the wings were cut from the thorax and placed on a glass slide in a drop of isopropanol. The wings were arranged on the glass slide to have same orientation. After isopropanol evaporated the wings were mounted in 10µl of Euparal (Roth GmbH). While putting the cover slip care was taken that the flow of mounting medium is not against the normal axis of wing hair orientation. The samples were allowed to dry for few hours.

2.10 Microscopy, Image Acquisition and Processing

a) Confocal microscopic images were obtained using LSM Leica TCS SP2 or Zeiss LSM Meta system. The images were processed using Image J-1.29 and Adobe Photoshop.

b) Simple light microscopic images were captured using Zeiss Axioplan2 microscope using standard optics with 10X or 20X PlanNeo objective lenses with help of IPlab 3.5 imaging software. The images were processed in Adobe Photoshop.

2.11 Primer designing, PCR and DNA sequencing

Primers were designed using MacVector 7.0 software (Oxford Molecular Group). The DNA was PCR amplified using Taq polymerase. The sequencing was done at the MPI-CBG DNA sequencing facility.

2.12 Plasmid DNA isolation from bacterial culture

For isolation of plasmid DNA from bacterial culture, simple and crude mini-prep method was used. A single bacterial colony was inoculated in 2 ml LB medium containing appropriate antibiotic and was allowed to grow overnight at 37^0C on a shaking platform.

1. 1 ml of the culture was collected in an eppendorf tube and spin at 5000 rpm for 1 min to pellet down the bacteria.
2. The supernatant was discarded by decanting and the pellet was resuspended in 100µl of LB.
3. Bacteria were lysed using Standard alkaline lysis protocol and proteins were precipitated by adding 350µl of 3M Sodium acetate.
4. After spinning at 13000 rpm for 15 min in a tabletop centrifuge the supernatant was transferred to a fresh tube and the DNA was precipitated by 2 volumes of cold 100% ethanol.
5. The DNA pellet was collected by spinning the sample at 13000 rpm, 15 min at 4^0C.
6. The supernatant was discarded and the DNA pellet was washed with 500 µl of 70% ethanol, air dried and then dissolved in 50µl of TE.

The quality and quantity of DNA is good enough for carrying restriction enzyme digestions. For sequencing purpose the DNA was column purified (Quiagen).

2.13 RACE analysis of CG31306/ *bitesize*

Total RNA from 10 adults was isolated using TriZol based protocol and was resuspended in 10μl of DEPC-H2O. 5 μl of total RNA was used for first strand synthesis reaction. The Superscript reverse transcriptase (Invitrogen) was used for first strand synthesis reaction primed with GSP1. Herculase® Enhanced DNA Polymerase (Stratagene) was used in the PCR reaction primed with the 5' FYVE 3507-25 and 3' GSP2 primer. The product of the reaction was cloned using TOPO® Cloning vector (Invitrogen).

The second RACE reaction was performed using the SMART™ RACE cDNA Amplification Kit (BD Biosciences) and the PCR reaction was performed with Advantage™ 2 PCR Enzyme System.

2.14 Bacterial transformation

Ca^{2+} competent XL1-blue or DH5α cells were used for transformation. All steps were carried on ice unless mentioned otherwise.

1. The bacterial cells were kept on ice for thawing and then 100μl of the competent cells were transferred to 15 ml FALCON tube chilled on ice.
2. 50ng of the plasmid DNA was added to the cells and mixed by tapping and kept on ice for 25 min.
3. A heat shock at 42^{0}C was given in a water bath for 45 sec and the tube was immediately transferred to ice for a 2 min cold shock.
4. 1 ml of SOC medium preincubated at 42^{0}C was added to the cells and then the cells were allowed to recover at 37^{0}C on a shaker at 200rpm.
5. Bacteria were collected by spinning at 5000 rpm for 1 min and the supernatant was decanted. The bacteria were resuspended in 100ml of SOC and plated on LB plate with appropriate antibiotic. The plates were stored at 37^{0}C overnight fro colony growth.

2.15 Primers used

Primer name	Sequence 5' to 3'	Application
GSP2- 6990-68	CAA CTG CTG CTG ATG CAC TTG GA	RACE
GSP1- 7031-12	TGT TGC AGG TGT GTC AGA TG	RACE
btsz-537	ATG CAG CCC AGT CAA CAA CAA CAA C	RACE
FYVE 2008-25	GAG GCA CCG AAC TGG GAA	RACE
FYVE3551-28	TTC GCC GTG ACA GGA CGA CGA TGA	RACE
FYVE 3507-25	CTC GTC TTT CTC AAC TCG G	RACE, sequencing RACE products
FYVE 5013-30	CAA GTA CCA GCA GCG GCA	sequencing RACE products
FYVE 5121-02	CAG CAT TTT CGA CTG CAA GT	sequencing RACE products
FYVE 6504-22	GAC AAC AGT GAC TAC TCG C	sequencing RACE products
FYVE 6616-99	GGT AGG CGA GCA CTC TCT	sequencing RACE products
FYVE 7991-10	GAG AAA GCG ACT GGT AAG AG	sequencing RACE products
FYVE 8096-78	AGA AGT AAC TGT GGC ATC C	sequencing RACE products
FYVE 9470-87	ATA AGA ATG CGG CCG CAT TCG CAT CAC CT	sequencing CG31306 cDNA
FYVE 10329-47	CTC TAC TCC AAA CGC GAT G	sequencing RACE products
Btsz-Ab2-10374	CAT CCA TGG AGG AAG CCA AAG TGG CCG	sequencing CG31306 cDNA
DBD 1494-71	TAA GAG TCA CTT TAA AAT TTG TAT	sequencing Y2H constructs
AD 2068-47	CGG GGT TTT TCA GTA TCT ACG A	sequencing Y2H constructs
T3	AAA TAA CCC TCA CTA AAG GG	sequencing
T7	GTA ATA CGA CTC ACT ATA GCG C	sequencing
M13 Rev.	CGA AAC AGC TAT GAC CAT G	sequencing

Chapter III: Results

3.1 Objective

As mentioned earlier the main objective behind this project was to identify proteins interacting with Widerborst and characterising an interacting partner affecting aspects of planar polarization in wing (hair polarity and/ or hair morphogenesis). In a genetic interaction screen based on EMS mutagenesis to isolated suppressors of over expression phenotype of Wdb (wing hair duplication) resulted in 5 wdb alleles (Hannus et al., 2002). Since this approach failed to identify genetic interactions I decided to carry a Yeast Two Hybrid screen to identify direct protein-protein interaction.

3.2 Yeast Two Hybrid Screen To Identify Wdb Specific Protein-Protein Interactions

The Yeast Two Hybrid system provides a sensitive method for detecting relatively weak and transient protein-protein interactions that might not be covered by biochemical techniques such as Immuno-precipitation.

I used the MATCHMAKER GAL4 Two-Hybrid System (for details see Material and Methods section). Full length Wdb protein was used as bait and the home made 20-22Hr-apf (After Puparium Formation) cDNA library was screened against. I decided to use this time frame for synthesis of the pupal enriched cDNA library keeping in mind that the machinery required for planar polarization and hair morphogenesis might be available at this stage in form of mRNA. Before starting the screen, the yeast cells were transformed with pGBKT7[wdb] construct and expression of the N-myc tagged DBD-Wdb fusion protein was confirmed by western analysis (Fig.9).

After screening approximately one million independent transformants, followed by reconstitution the number of confirmed positive interactions was reduced to 37 cDNA fragments. After sequence analysis of the cDNA it was revealed that these 37 fragments represent open reading frames of 18 different genes. These included

variety of different proteins. At least 8 were novel proteins at the time when the screen was performed. The rest included transcription factors, RNA binding proteins, putative trans-membrane proteins and proteins with no specific domain. Cyclin-G was one of the most frequently isolated candidates and is already known to be interacting with B' type of regulatory subunits of PP2A (Ito A et al., 2000). The information is summarized in Table 1.

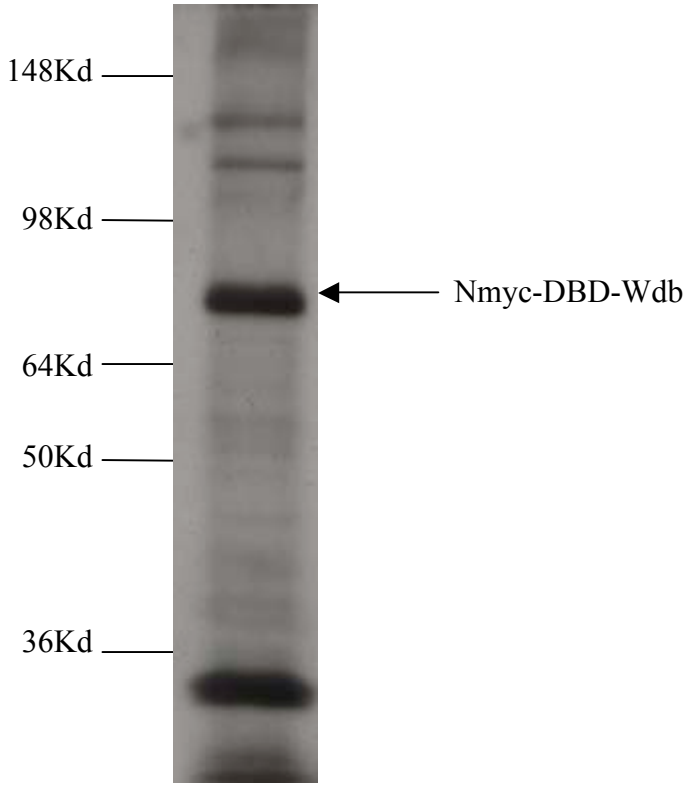


Fig 11: Western blot for checking expression of the bait protein in yeast: extract form 10µl of yeast cells expressing the Myc tagged bait protein was 50µl of reducing lamelli buffer and 10 µl of the lysate was loaded. The blot was probed with Rabbit anti Myc primary antibody and the signal was detected with HRP tagged anti-rabbit secondary antibody after ECl reaction.

Table 1: Summary of the candidate proteins interacting with Wdb isolated in the Yeast Two hybrid screen.

No.	Gene	Protein and Size (aa)	# of times Isolated	Specific domains (SMART analysis)	Predicted/ known Molecular function	Clone Id	Smallest fragment (aa onwards)
1	CG11525	Cyclin G (566 aa)	7	Cyclin	Cell cycle dependent protein kinase	W24	200
2	CG134933	Cookie monster (600 aa)	4	-	Meiotic cell cycle arrest in spermatogenesis	W48	START
3	CG8566	Tantalus (299 aa)	2	-	Sensory organ Precursor development	W65	31
4	CG6315	female lethal d (536 aa)	1	-	Female germ line sex determination	W17	143
5	CG5836	SF1 (787 aa)	1	KH domain CCH type Zn finger	Splicing factor	W53	205
6	CG10119	Lamin C (621 aa)	1	-	Nuclear envelope assembly	W18	280
7	CG17958	Serendipity δ (433 aa)	1	Zn finger	Transcription factor	W3	171
8	CG2467	Novel (963 aa)	4	TM, ZP like	?	W8	722
9	CG7574	Bip1(419 aa)	3	-	?	W52	163
10	CG10173	Best 2 (731 aa)	2	-	?	W31a	274
11	CG9188	Septin interacting protein 2 (625 aa)	2	-	Septin interacting	W1b, W10b	20
12	CG4400	Novel (259 aa)	1	-	Similar to Breasts cancer metastasis suppressor	W23	START
13	CG8145	Novel (370 aa)	3	C2h2 Zn finger	RNA binding	W36	START
14	CG1553	Novel (834 aa)	1	-	?	W27	277
15	CG18768	SP2523 (1716 aa)	1	-	?	W35	1189
16	CG14561	Novel (191 aa)	1	-	?	W15a	31
17	CG3259	Novel (588 aa)	1	-	Ortholog of Microtubule interacting protein T3	W2a	349
18	CG31306/ CG7343	Bitesize	1	C2	?	W9	2133

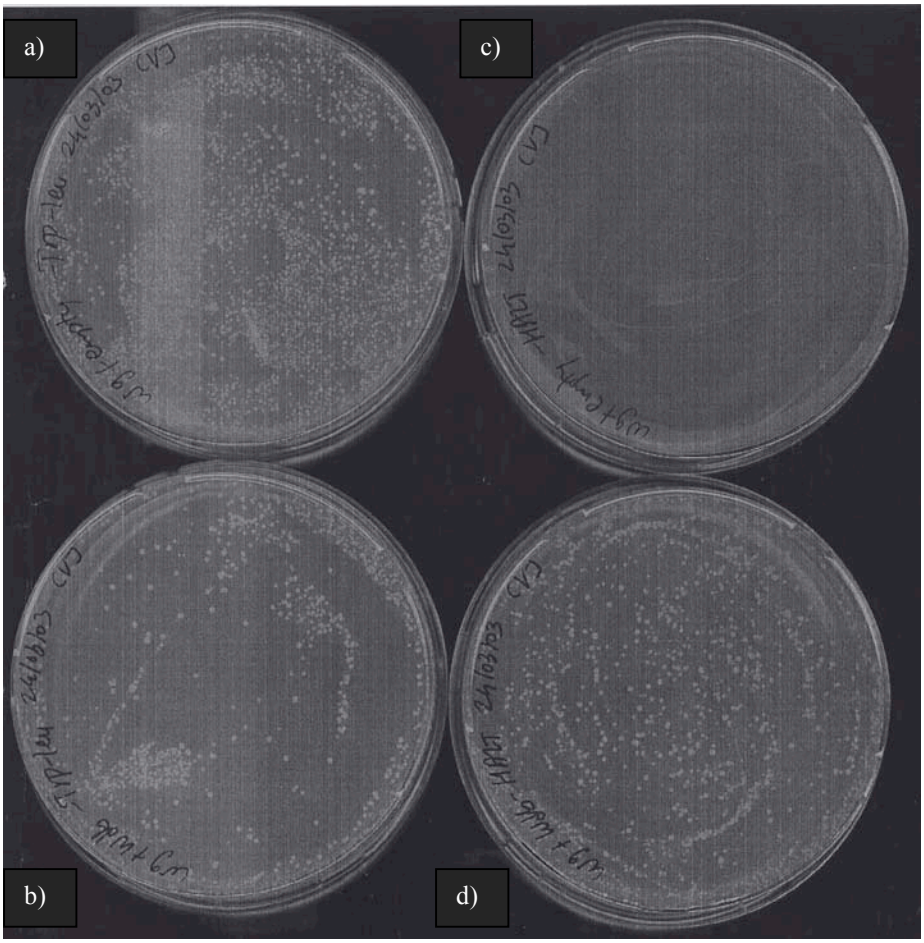


Fig.12: Representative Reconstitution Assay result: Each candidate was subjected for reconstitution assay to check its interaction with empty bait vector pGBKT7 in yeast strain AH109 and was grown on selective growth medium to get rid of false positives. Four day old plates from reconstitution assay for clone W9 are shown here. a) pGADT7[W9]+ pGBKT7[empty], b) pGADT7[W9]+ pGBKT7[Wdb], c) pGADT7[W9]+ pGBKT7[empty], d) pGADT7[W9]+ pGBKT7[Wdb]. a) and b) were grown on -Trp-Leu medium to check transformation. c) and d) were grown on –Trp-Leu-His-Ade medium to confirm interaction.

Since the Yeast Two Hybrid screen is based on protein to protein interaction and not on phenotypic class, it is important to analyse the mutants whenever available for the specific phenotype of interest or to carry a secondary genetic interaction screen as a supplement. The following considerations were taken into account for selecting a candidate from further analysis with respect to planar polarity:

1. The predicted/ known molecular function.
2. Screening of available mutants for planar polarity/ wing hair morphology defects.
3. The over-expression phenotype of Wdb in wing is hair duplication. Available deficiency stocks for the candidates from the two hybrid screen were collected from Bloomington and were crossed with a stable Wdb over-expressing and the wings of the F1 progeny were checked for suppression of wing hair duplication. Such strategy allows to isolated dominant suppressors of Wdb dependent wing hair duplication.

Based on the first approach initially the main focus was on two candidates CG3259 and CG31306.

CG3259:

CG3259 is an ortholog of mammalian Microtubule Interacting Protein T3 (MIPT3). Because of microtubule localization of Wdb and the in general significance of microtubules in wing hair formation, CG3259 was of interest.

The C-terminus of mammalian MIPT3 is known to interact with DTRAF1 while DTRAF1 is known to be involved in direct interaction with Ste20 kinase Misshapen. Interestingly Misshapen is required for ommatidial polarity in the eye and also for wing hair morphogenesis.

However due to lack of genetic reagents, investigation of role of CG3259 was not possible.

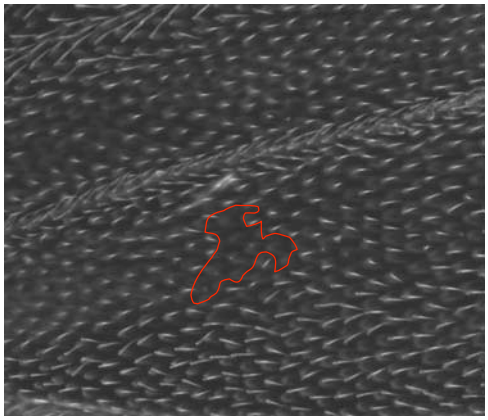
CG31306/ bitesize:

The predicted sequence of CG31306 has an N-terminal FYVE finger domain. Proteins with FYVE finger domain play significant role in regulation of endocytic machinery (DiNitto J et al., 2003). Since endocytic machinery and vesicular trafficking is thought to play role in regulation of planar polarity, a FYVE finger protein interacting with Wdb provided good enough interest.

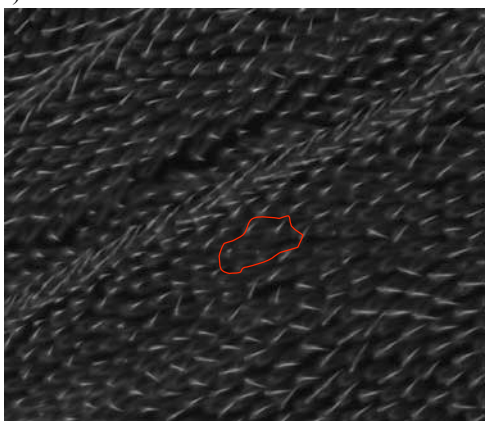
3.3 CG31306 Mutants Show Wing Hair Defects And Interact With *wdb* Genetically:

Two different P element insertion lines were available for CG31306, an EP element insertion (EP3567) line and a PZ element insertion (BL11743) line. Upon inspection it was observed that the homozygous wings of the PZ insertion line BL11743 show stunted wing hairs particularly in the central region of the ventral wing surface. No defect was observed in wing patterning and morphology. These P-element insertion mutants are recessive alleles since removal of only one copy of the protein was not sufficient to give the truncated wing hair phenotype.

a) BL11743/BL11743



b) BL11743/EP3567



c) wdb^{14}/ wdb^{dw}

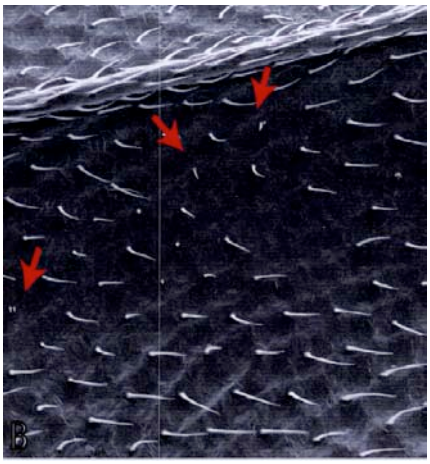


Fig. 13: a) BL11743/BL11743 homozygous wings show bald patches or stunted hairs. This phenotype is evident on the ventral surface of wing, especially in the central region. b) The heterozygote wings, btszEP/btszPZ also showed same kind of phenotype. Images were taken using dark field condenser and processed in Adobe Photoshop. c) Scanning electron micrograph of wdb^{14}/ wdb^{dw} wing. The arrows mark truncated wing hairs (From Hannus et al.,).

This weak truncated/ bald patches wing hair phenotype observed in CG31306 mutants was similar to heteroallelic phenotype in wdb14/wdbIP wings. Due to this similarity in phenotype I checked for possible genetic interaction between Wdb and CG31306. Bald or patches with truncated wing hairs were observed on wings form trans-heterozygote flies, indicating that CG31306 and Wdb interact genetically with each other. More ever, these patches were not restricted to central area of ventral wing surface but could be observed in any region of the wing. Also the size of patches was bigger indicating that removal of one copy each of wdb and CG31306 was sufficient to cause stronger phenotype.

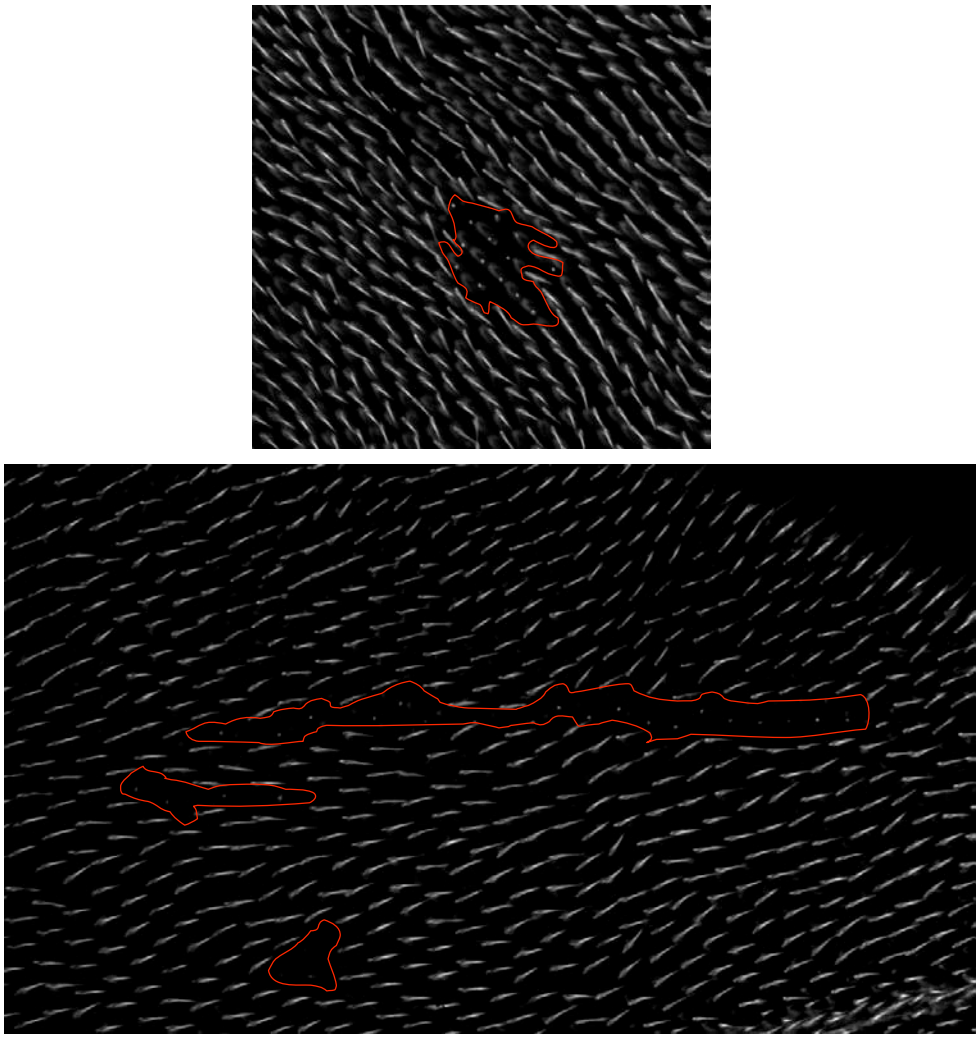


Fig. 14: CG31306 genetically interact with wdb: Dark field microscopic images of wings from wdb^{14}, + / +, EP3567 flies are shown here. The wings show bigger patches of truncated wing hairs than those observed in homozygous mutants of CG31306.

The EP element lines are traditionally useful for GAL4 dependent over-expression of the trapped downstream gene. However the nucleotide sequence flanking EP3567 provided by flybase indicated that the orientation of the EP3567 is opposite to the open reading frame of CG31306. Hence EP3567 couldn't be used for over-expressing CG31306.

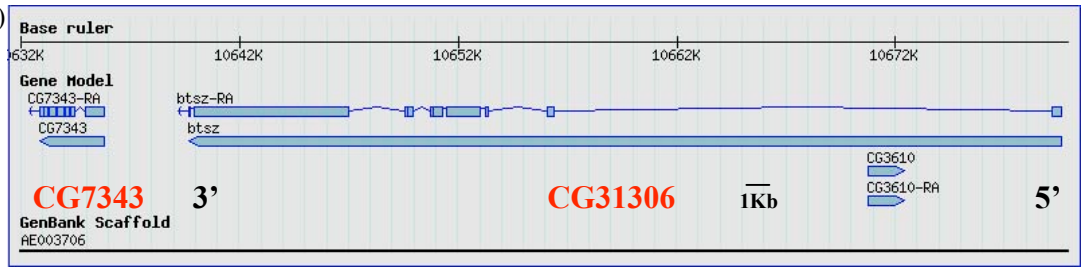
Overall taking into consideration this data with the Yeast Two hybrid interaction, it can be said that wdb and CG31306 genetically interact with each other and can be involved in direct protein-protein interaction to regulate hair morphogenesis.

3.4 W9 cDNA Codes For A Protein That Is Fusion of CG31306 and C terminal of CG7343 With Tandem C2 Domains In Its Terminal

The CG31306 transcription unit covers approximately 45Kb region and is predicted to have at least 9 exons. The Drosophila genome annotation release 3.1 predicted a 10406bp mRNA from CG31306 with 3289aa protein product. The cDNA fragment of CG31309 isolated in the Yeast Two Hybrid screen was of approximately 5.5 Kb. To analyze the DNA sequence of the fragment I designed sequencing primers based on the sequence provided by Flybase genome annotation for CG31306. The cDNA was PCR amplified and the PCR product was sequenced. The nucleotide sequence suggested that the cDNA fragment W9 matched with the predicted mRNA sequence from 6932-bp onwards till 10374-bp. However the rest of the C terminal region of the W9 cDNA fragment didn't match with the predicted C terminal region of CG31306. Instead the nucleotide blast suggested that the remaining C-terminal portion of the W9 cDNA fragment correspond to the C terminal of adjust gene CG7343. This data is summarized in Fig. 13.

The C-terminal shared by CG7343 with W9 cDNA codes for conserved tandem C2 domains. Thus the protein coded by W9 cDNA fragment isolated in the Yeast Two Hybrid Screen is hybrid of C terminal region of CG31306 and C terminal region of CG7343 with tandem C2 domains. Over all this suggested that the genome annotation predicted for CG31306 and CG7343 is wrong and that they might be part of a single open reading frame.

a)



b)

Predicted mRNA length for CG31306	10406 bp
CG31306 mRNA region matching with W9 cDNA	From 6932 bp to 10373 bp
Predicted mRNA length for CG7343	2127 bp
CG7343 mRNA region matching with W9 cDNA	From 856 bp to 2127 bp

c) Diagrammatic presentation of nucleotide sequence alignment of W9 cDNA fragment.

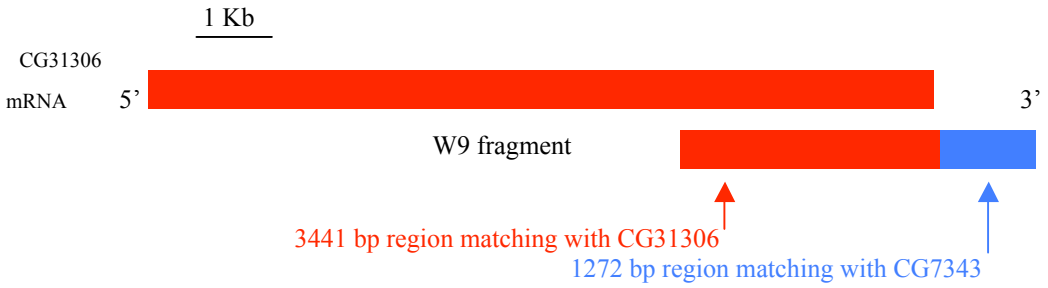


Fig 15: a) Annotation of chromosome 3R:10632k-10679k region as predicted by Flybase. The approximately 48-Kb region was predicted to include two genes namely Cg31306 and CG7343. The cDNA fragment (W9) isolated in Yeast Two Hybrid screen is fusion of CG31306 and C terminal region of CG7343. b) Table summarizing the nucleotide sequence alignment analysis for W9 cDNA fragment. c) Diagrammatic presentation of the W9 cDNA fragment showing region aligning with predicted mRNA of CG31306 in red and region aligning with predicted CG7343 mRNA in blue. The rest of the portion of the W9 cDNA didn't generate good quality sequence.

W9 cDNA Sequence: Length: 4713 bases, Orientation 5′ to 3′

```
   1  TTCGACAAAA TATTTGGTAG TCATCAGCAA AACACTCCAA GTGCATCAGC
                                        3′AGGTT CACGTAGTCG  Primer GSP2

  51  AGCAGTTGCA GCAACAGCAG CAGCAGCAAC ATCTGACACA CCTGCAACAT
      TCGTCAAC 5′                   3′G TAGACTGTGT GGACGTTGT 5′ Primer GSP1
 101  CGCCCAGCGA ACTGGCTTCG CCCAAAAATT CACTTTCAGC CAAGTATCGC
 151  AGTTTGGTCA TGATCAACAA AGACAATGAA AGTGCAGCTG GCGATTACGA
 201  AATGGCAAAC AGCAGCAGCA ACTTCAGCAA CATCAGACAC AAAGACCCGA
 251  CCAGTAGCAA CAACAGCCAC ACTGTTGGCC AGAACTTTGC GAGTGACTCA
 301  TTTCGCGCCA TGGAACCAAC GGAACTCGTG AGCAGCGATC GCGAAGCGTA
 351  CAGTGTGGAC TCGCTAAACG AACCGCCTGC TGTGCCGGAA CTCAGCGTAG
 401  AGGACGGCTT GGCTGATGAC GACTCCTGGG TGGAGGAACT GAGTCAGCGC
 451  GGCGAGGATG AGGACGAAGA TCTCAGCAAT GCTACCACCA CACCCACGGC
 501  CACAGACTCC GAAGATGCGG AGGGAGAAGG TGAAGGAGCC CGGCGACATG
 551  GATATATGGA TCGAGAGGAG GACCTAAGGG GATACAATAG ATCTGCCATC
 601  GACTTCACCC TGCACACCAT CGTCGAGGAG AGTTGTGAGG AGAGCGAGGT
 651  GGCCTCCATG CGGGCGGACA ATGAAGACGT CGAGCTGGAG GATGAGGATC
 701  TATCCGAACG GAGAAGGCAG CGCACCTTGC AGCACCACCA TCGACTAAGT
 751  GCCTCTGAAC TAGAGAAGTA CTTCTTCTTT GGGCTGGGAG ATGGTCGAGT
 801  AATGAGTTCT ATTGATACAC GCGGAGATGA CACTGCATCC GAAGTGAGTT
 851  CCGAGTGCTC TGAGAGTCTT GACTCCCTAC CGCATGAGGA TCAGCTGTTG
 901  GATACAAGTG GAGCTTCCGA TCTGGCCTCT TCCCGTCTGG AAAAGTACTT
 951  CCTCTCCGGC TTCATGGGAT TCAGTGGTGC AGAAAAGCAG GCGGAGAGCG
1001  ATGAAAGTGG TGGCAGTGTA GGCAGTGATA GTGAAGGACA ACCGAGTCCC
1051  AGTCAGCGGA GAAAGCGACT GGTAAGAGCC AGGGGAACAC CTAGAAGTCA
1101  CAACTCCTCG TTGGATAATT TGCTACTGCC AGAATCAGAC ACCCTGGATG
1151  CCACAGTTAC TTCTGCAGGC GGAGCAGCTG CAGCCACTGT AGAGGACACC
1201  TCGGAATCGG AGGCGGGCTG TGACGACACT GTGATTCATT TGGCCAATGC
1251  CGGAGCTTCG GATGGTTCCT CCTCGGACAC CATTAAACGC AAGAAGCAAC
1301  TGCGCAAGCG TCACGACTCT CTGGACGAGA AGAAGCTCCA CGATCTAGAG
1351  CCTTCCGAGT GCCGGACTCC CACCCCGGGC AGCAGTGGTC AAGTGCAGAT
1401  GCAGTCGCAG GCGAAAAAGC AACAACAGCA GCATCACCAC AGCCGGGACA
1451  GTGGCTTTGT GGGCAGCAAC GATGATCTCC TGAAGGCGGC ACCAGATTGT
1501  GAGCCACCCA AGAGCCCAAC GCCAGCTCTG GAGCAGATCA GTGAGGATCG
1551  GGAACCGGTC CATAGTCAGA TTAGTCTGGC CAGGATGGAT CAACCAAGCA
1601  CTTCAGCAGC TGCAGCTGTA GCCAGCACAC TTTCCAGCCA ACTGAAAAAT
1651  CTTGCCCTGC CCAATCTGGT GCGAAAGGAT AGCTTCAACA ATTGGAGCTC
1701  CGACGAGGAG ACCAACCTGA TGATGAGCAA GATGCGGCAG TTCTTTAAGA
1751  ACCTCATTGT CGCCACCGCC AATGCACAGC AGAGCAAGCC CTCCACGCCC
1801  AATCAAGGGC CCAGCACCAC CAATGTCAAC ACCACGCCCA GTTCCCAGCG
1851  CAGATTGGCC AAATCCCGGC CAGCTCAGTT GGCCTACTTT GAGAATGAGC
1901  TGACCCGACT GATGAAAACC GTGCCGGGCA TCAATGATGA GCAGGTGCGC
1951  GAAATAGTGG AGTACCTGAG CAGTGAGGAT ACATGGTCTG ATTCCTACGA
2001  TTCTTCGGAC TACACGAGCT CCGATCTGGA GGGCGGTGAA AGGAAGGGCC
2051  AACTGAAGGC TCAGATCTCC GCCAGCTGTC AGCAGATCAT CAACAAGTTT
2101  GAAATTGACG AGGAGGGTGA TCGTGGTGAT GGTGGACTGC TGGACGAGAG
2151  CCAAAGTGTG CCTATGGAAG CATTGGTGTA TCAGAAATTG GTAGCTTCCT
2201  TTAGCAAAGT GGCGGCGGGT GGAGAACCGG AAACAGAAGC CACCAAAGCG
2251  GTGGAGGAAG CAGAACCGTC CACGGAGAGA TCCCCGCAAC TGTTTGCCAA
2301  GGTGATGCAG CATATTGGTA CCAGATTGGT AGCTCTGATG CACGAAGTTA
2351  GCAGTGGCAA TGAAACACCC ACTCCATCCC CGCAAGGACA GCGGCATCAT
2401  CGAAGACTGC AGGCCAAGAT CTCGGCCACC ACGACGGAGG ATGAGGAAGA
2451  TGAGGTGGCG GAGCAACTGA AAGCCATGCC CATCAAGCAG CTAAAGCTGC
2501  GAAGCAAATC CCATGATCTA CTGCTGGACG GCACTACGCC GCATTCGCAT
2551  CACCTGCACC ACCATGCCAC GGTGCATTAT CCAGCCGGCG GCGTGGGATC
2601  GGGAGCGGGA GGATCCGGCG GACCCGGACA CTCGGACAAC GCCGGGGAGG
2651  AGTGCGGCGT GACCAGCGAC TACGAGCGAT TCTCGTGGCG CGGCAGTTTT
2701  GAGTCGGCAT TACTGGCTAA TGGCGACAGC AGAACGAGGC TCAGCCAGCT
2751  GAGCCAACTG GACAGGGATA ACTCGTCGTC TGCGTCCGCT TTGGCTGTGG
2801  CAAAGCGCCG ATCGGCAGGC GACCTTCTCT TCAGCCAGCA CCAGCAGAGC
2851  CTGAGCCGCG AGCAATTGGA TCGCGTCCGA TCCTGCGGCA GCATTGGAGG
2901  CGGCGATGCC CATCACCACC AGTTGGAGTC CTCGCCGGCC AAGCCGTGGC
2951  TCTCCTCCGC GGGATCCTCG ATTGGCGGTG ATTCTACGAA GGATGTGCGG
3001  CGTTCCAGTG TACCGGATGC CATTTACGAG ACGGACTCCA GCGATGAGGC
3051  AGCTTCGCAT CAGTTTGGCG GCGCCAGATC CACGTTGCCG CGCAGTCTGA
3101  ATCCCAACCA AGTGGTGGCT AGCACGAATT CACTACCCCG GTTACCCACC
3151  ACCGGTGTTG GTGCTCCCAT AACCAGCACC CCCAAGACAA AGTCTCAGAG
3201  CGCCCTCAAC CACACGCCCT CTAATTTGTC CACCGTTTCG GCCACTGGCA
3251  GTGCCAAGAG TGCGAGATAC CGCTCGCCAG GATTGGCAGC TCGAGCAGCG
3301  GCCGTCAGTG GGGCAGGAGG TGGCTCAAGT GGTGGTGTTG GTGTGGGTGC
3351  CAGCAGTGGT TCGACGGGCA AAAAACTAGG CGCGGGCTTC CAGTTCCTCT
3401  ACTCCAAACG CGATGCGCGC AAACGTCTCA ACATGTCAGC TGAGGAAGCC
3451  AAAGTGGCCG CTGAGGAACT GTCACGATCG CCGGTAATTG GACAACGGCA
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3501  GGCAGATGGA TCGGGCAGTC CCATTCAGTC GCGTGCCTCC AGCGAAACGT
3551  GGCCCACTCA GTCGGATGAG GACATCGATC GCCTGGTGGC CATGCATCAG
3601  AACCGCAGCA GTCTCAGCTC GCTGGGGGTT CGATCCGAAT CTATGGCCAG
3651  CGTTTATTCG GGCGCCGGAG AAGGTCGCTA TGGCACCGTG GTGGTCAAGG
3701  GTCAAGTAGA GTTCGCCATG CAGTACAACT ATAAGCTGAG TGCTTTGGAA
3751  GTGCACGTGG TTCGCTGCAA GGACTTGGCT GCCGTCGATG CCAAACGCAA
3801  TCGCAGTGAT CCCTACGTTA AGGTGTATCT ACTGCCCGAC AAATCGAAGG
3851  CTGGCAAGCG CAAAACCAAA GTCAAGAAGC ACACGCTGAA TCCCATCTTC
3901  GACGAGACGA TGCGCTTCCA CACACCCATT TCCAGCTTGG AATCACGAAC
3951  ATTGTGGCTC ACCGTCTGGC ACTCGGATAT GTTTGGGCGT AACGACTTCC
4001  TGGGCGAAGT CAGTGTCAAT TTACAGGGGC GGCTCTTCGA CAATCCCCAG
4051  TCGCAGTGGT ACCTTCTCCA AGAACGCAGC GAACCCTTCG ACGAGGTGGC
4101  TACCTACCGA GGTGACATCG TAGTGGGTCT GAAGTACATT CCCCCAGAGA
4151  ACATCAAATC CTCGTTCTTT TCGCGCGGCT CCTCAATAAC AGGCAGCTCC
4201  TCGAACTTGC GCAAATTTGG AGGCAGCATT AAGTCGGTGG CCTCCAAATC
4251  AGAACGCACT TCAAAAGGCG GCCAGCTGCA TGTGCTGGTC AAGGAGGCCA
4301  AGCATCTGAG TCCTATAAAG GCGAACGGCA CCTGCGACGC CTTCTGCAAG
4351  AGTTATTTGT TGCCCGATCG GACGAGGAGC TCCAAGCAAA AGACGCCGGT
4401  GGTGAAGCGC ACCCTGCATC CCAGCTGGAA CTACACATTT GTTTACGAGG
4451  ACGTCTCCCT GGAGGAATTG TCAGAACGCG CCTTGGAGCT AACCGTCTGG
4501  GATCACGATC GTTTGGCCAG CAATGAGTTT GTTGGCGGCA TACGATTCTC
4551  GTTGGGAACC GGTCGCAGCT ATGGCCGGCA GGTGGAATGG ATGGATGCCA
4601  CTGGAAAGGA GCTCTCCCTG TGGCAGAACA TGTTGGATCG TCCGAACTTC
4651  TGGGTTGAGG GCAGCTTGGT TCTGCGTTCC AGTTTGGATG GCATTCGAGC
4701  CAATTTGCCA TAG
```

Fig. 16: Nucleotide sequence of W9 cDNA: The red region belongs to CG31306 and the blue region belongs to CG7343. The 5'UTR region is not provided here. The region complimentary to GSP1 and GSP2 primers used in RACE analysis is underlined and the actual primer sequence is shown in black with the orientation. GSP1 was used for first strand synthesis in an RT-PCR reaction and the second strand was synthesized in a PCR reaction with primer 3507.

Keeping the possibility in mind that the W9 cDNA fragment isolated in the Yeast Two Hybrid screen might be incomplete I decided to look for the rest of the N terminal region of the predicted CG31306 mRNA.

3.5 5' RACE (Rapid Amplification of cDNA Ends) for CG31306:

The 5' RACE analysis resulted in an approximately 3.5 Kb PCR product primed with following primer pair.

Forward Primer: [3507-25], CG31306 specific primer
Reverse Primer: [6990-68] GSP2

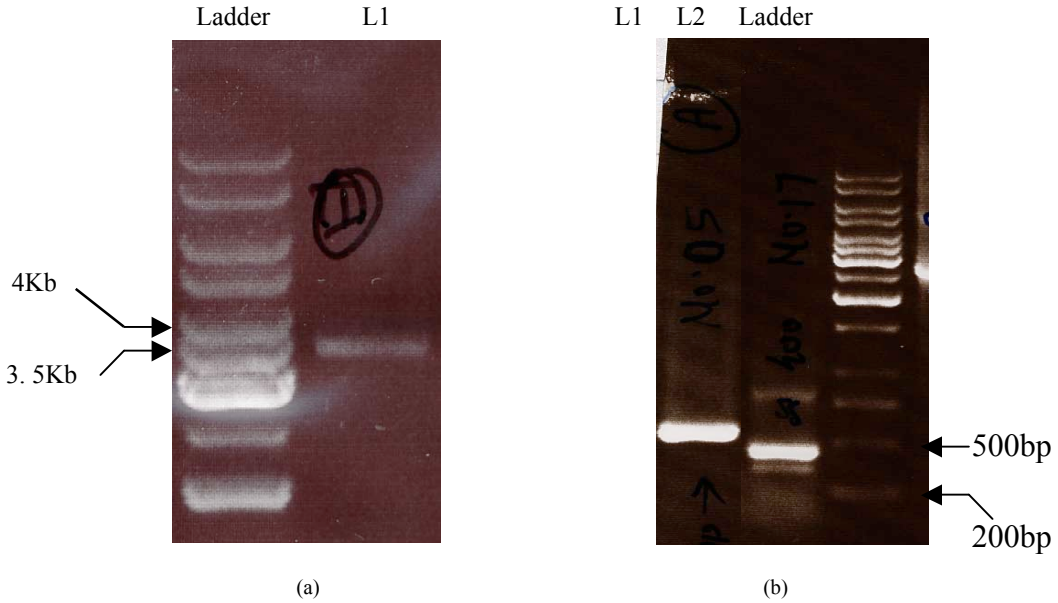

Fig. 17: 5' RACE analysis of CG313106: (a) A product of approximately 3.5 Kb was obtained from a 5'RACE reaction primed with forward- 3507 primer (based on predicted CG31306 sequence) and reverse GSP2 primer. (b) RACE reaction was carried out using SMART RACE kit (BD Biosciences) which facilitates reaching the N terminal of the cDNA with the 5' Short Up primer and the products were cloned in TOP O vector. L1 shows RACE-5 clone product obtained in a 5'Short Up-3' GSP2 6990 primer reaction. L2 shows RACE-17 clone obtained in a 5' Short Up-3' 3507 primer reaction.

The PCR product was cloned in TOPO vector and the sequence of the insert was analyzed. DNA sequence analysis revealed that this 3.5 Kb PCR product belongs to CG31306 and complements with the predicted region immediately lying upstream to the region obtained in W9 cDNA fragment.

In search of the remaining N terminal region of CG31306, I decided to use SAMRT RACE cDNA Amplification kit (BD Biosciences) which facilitates synthesis of full length cDNA. Two different set of primers were used for the RACE analysis.

a) RACE clone No.5 nucleotide sequence Length: 314

```
  1  AGTCGAAAAT GAAATGTTAA ACGTTGCGGT CGCTTCTCGT GCCGCGCTTC
 51  TCGGCAGCAA TAACAACAAC ACACACAGAA ACGATAATAG CAATAACGAA
101  AACACTAACA ACAACGACGG CGAACTTGGC CATAATAATA ATGACGCTTT
151  CGCAGCACAA AAGACGAGAA ATAGCGGCTC GAGTGTGGTT GTCGGCTGCG
201  ATATTAGTGA CGTAGTGAGT GTGAATTCAA ATGTGAGTGG TGAAAACGAG
251  TGTGACTTCG ACAAAATATT TGGTAGTCAT CAGCAAAACA CTCCAAGNGC
301  ATCACCAGCA GTTG
```

b) RACE clone No. 17 nucleotide sequence Length: 128

```
  1  TAGANNNCAG GTGTTCCACT CAGCACGGAC GGGCAGCAGC AGCAGTCGCA
 51  ATATAGCGTT GAAGCGGAAA NAGTTCAAGC TCGTCTTTCC CAACTCGGAT
101  TCATCGTCGT CCTGTCACGG CGAAAAGGGC GAATTCCAGC ACACTGGCGG
151  CCGTTACTAG TGGTCCGAGC T
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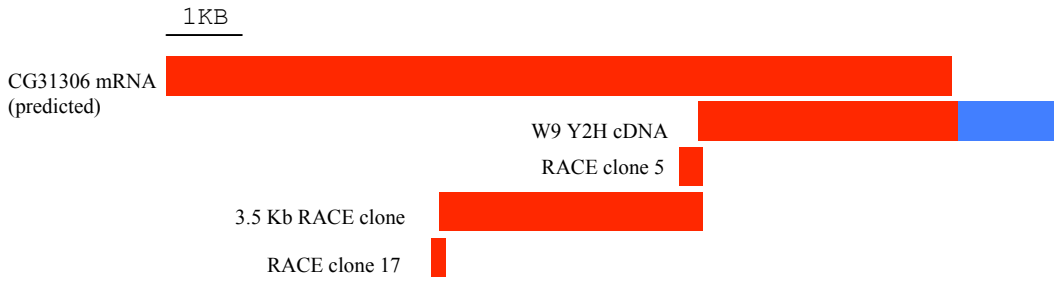
C) Summary of RACE analysis



Predicted mRNA length for CG31306	10406 bp
RACE clone 5, primers used for RACE	5' Short UP primer - 3' 6990 GSP2 primer
CG31306 region aligning with RACE clone 5	From 6667 to 6990 bases
3.5Kb RACE product, primers used	5' 3507 primer – 3' 6990 GSP2 primer
CG31306 region aligning with RACE product	From 3507 to 6990 bases
Race clone 17, primers used for RACE	5' Short UP primer - 3' 3551 primer
CG31306 region aligning with RACE clone 17	From 3430 to 3555 bases

Note: The numbers given in the table are based on the predicted mRNA sequence of CG31306. All primers used for RACE analysis were designed using the predicted mRNA sequence of CG31306.

Fig. 18: Summary of 5'RACE analysis of CG31306: a) Sequence of RACE clone 5. b) Sequence of RACE clone 17. c) Shows different cDNA fragments of CG31306 obtained from the RACE analysis and Yeast Two Hybrid Screen with their regions aligning with respect to predicted mRNA sequence of CG31306. The region compromised by CG7343 is shown in blue.

A] 5' Short UP and 3' GSP2 (CG31306 specific primer 6990): This reaction gave a product (RACE clone No. 5) that matched with region 6667-6990 on CG31306 mRNA (total 314 bp).

B] 5' Short UP and 3' primer 3551-28 (CG31306 specific primer): This reaction generated products of various sizes close to 100bp (+/- 25bp), matching in the region 3456-3550bp of CG31306. The sequence of the largest clone (RACE clone No. 17) is shown here.

Overall this suggested that at least tow different cDNA may originate from CG3106, one with its N terminal at about 6667bp and second at about 3456 bp (these numbers correspond to the predicted 10406bp mRNA for CG31306).

3.6 CG31306 is bitesize (btsz), A Protein With Tandem CT Domains

At the time when I was doing RACE analysis for CG31306, Serano et al (PNAS 2003) reported CG31306 as ***bitesize*** and isolated three different classes of transcripts namely btsz-1, btsz-2 and btsz-3.

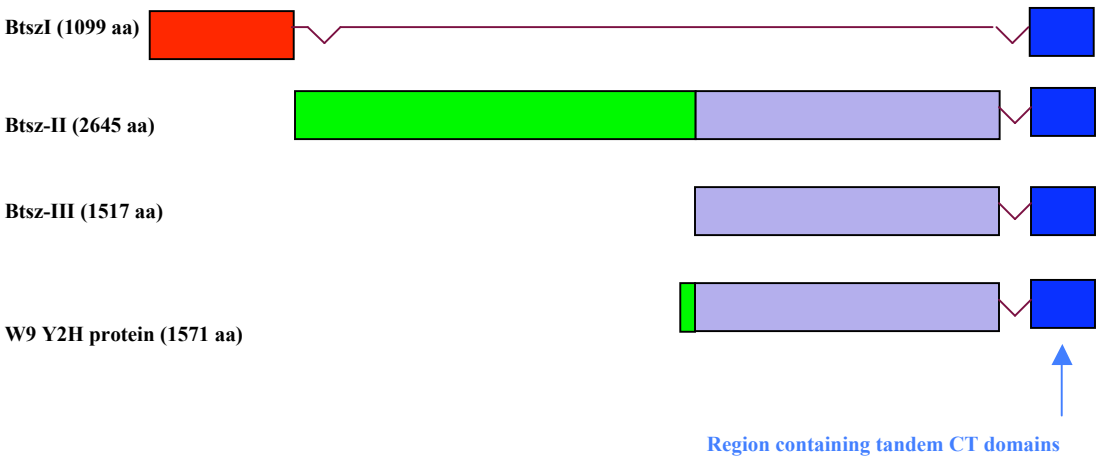


Fig. 19: Three are three isoforms of Bitesize protein. All of them share a common c terminal that contains tandem C2 domains. The protein coded by W9 fragment obtained in Y2H is compared here with the three predicted isoforms.

Fig. 19 shows the comparison of the three isoforms at protein sequence level. Btsz-I has a unique N terminal region (shown in red) and lacks the central region shared by Btsz-II and Btsz-III. The region shown in green is unique to Btsz-II. All the three isoforms of btsz share the C terminal region of 423 amino acids that codes for the CT domains.

The cDNA fragment isolated in Y2H screen essentially codes for the C-terminal 1571 aa region of btsz-II. Thus the region of btsz isolated in the Y2H screen is part of Btsz-II isoform though the possibility of Btsz-III interacting with Wdb can't be denied.

In the same publication Serano et al described P element excision mutation $btsz^{J5-2}$. $btsz^{J5-2}$ mutation is reported to contain a frame-shift mutation predicted to result in a truncated btsz-II protein of 393 aa, without affecting btsz-I isoform. This mutation results in flies with growth defects namely reduction in size. These defects can be rescued by ubiquitous expression on btsz-II. I decided to use $btsz^{J5-2}$ for analyzing the loss of function phenotype of btsz-II in pupal wings.

3.7 Mosaic Analysis of J5-2 mutant: Loss of btsz II Result In Stunted Hair Outgrowth

To analyze loss of function phenotype of btsz II mitotic clones of $btsz^{J5\text{-}2}$ were analyzed in pupal wings. $btsz^{J5\text{-}2}$ generates a truncated Btsz II protein and do not affect expression of btsz III. The clones were marked by loss of CD2 expression a membrane protein. The effect on wing hair formation was checked by marking the actin cytoskeleton with Texas Red-phalloidin. In clonal cells lacking btsz II function the wing hair outgrowths were less robust, indicating that btsz II function is important for proper extension of wing hair.

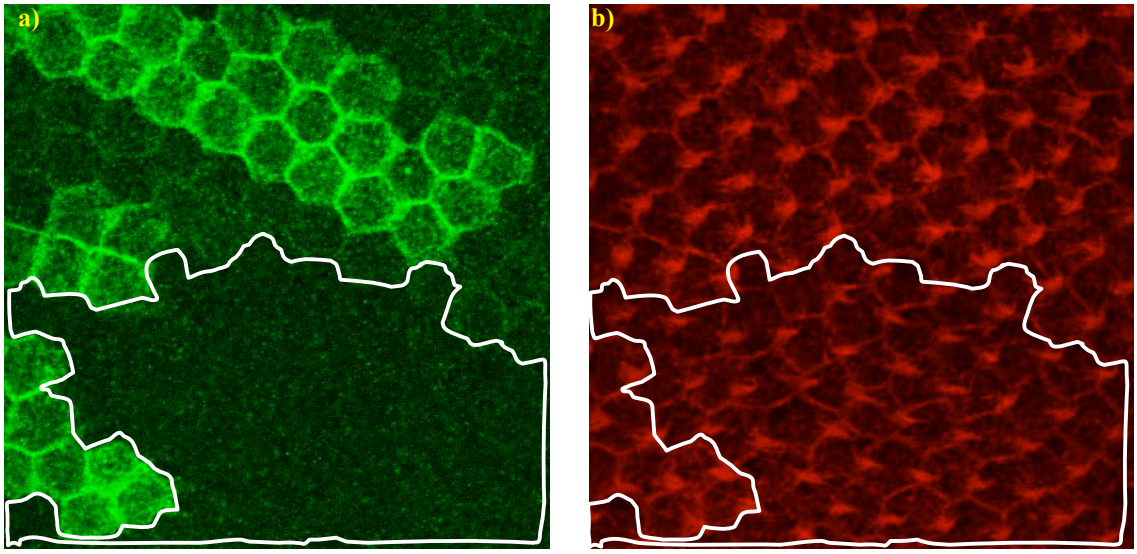


Fig. 20: Clonal analysis of $btsz^{J5\text{-}2}$: Mitotic clones of $btsz^{J5\text{-}2}$ were induced at late 3rd instar stage and the effect on hair formation was studied at 30Hr apf by marking the actin with Texas-red conjugated phalloidin. A single, apical confocal Z section is shown here. a) CD2 staining to mark clonal boundaries. The clonal cells were marked by loss of CD2 (green). The Twin spot cells are can be identified with strong expression of CD2. b) Phalloidin staining marking the actin (red). The clonal cells lacking btsz II are outlined with white boundary. The twin spot cells show accumulation of actin bundles filling the hair outgrowth at the distal age of the cell. The clonal cells show reduced level of actin filling the hair outgrowth specifically without affecting the cortical actin.

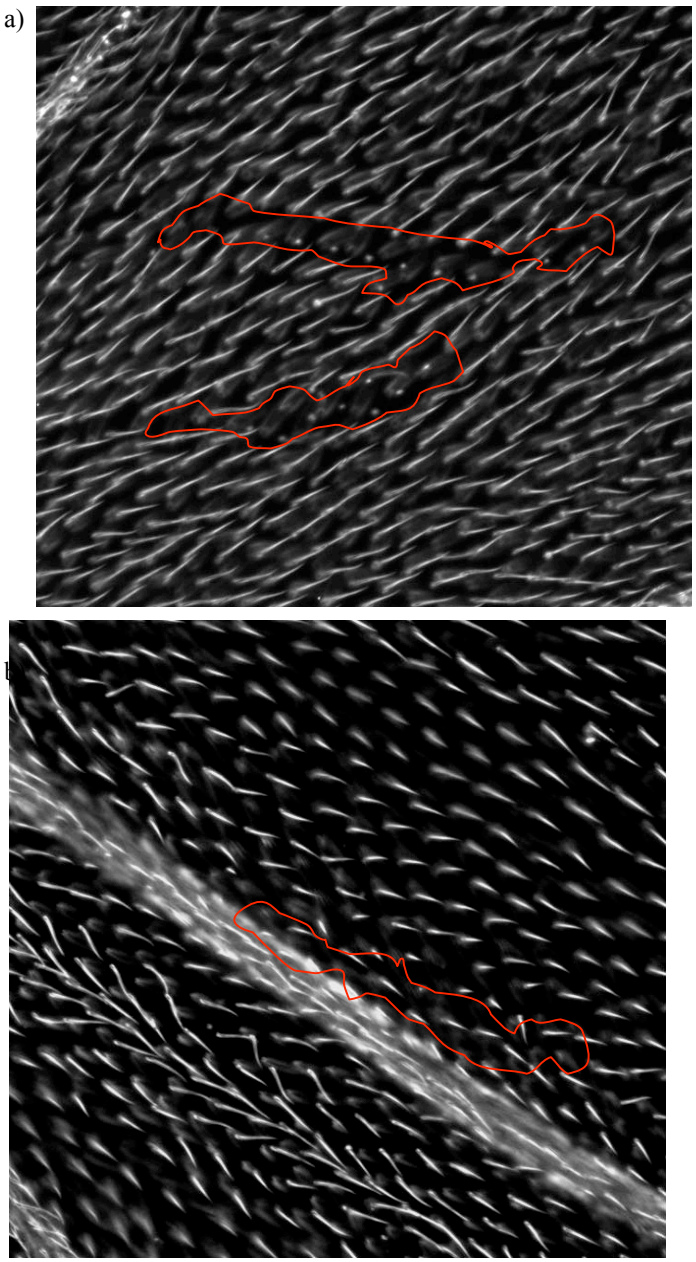


Fig.21 Adult wings with btsz$^{J5\text{-}2}$ clones show bald patches (a) or truncated hair growths (b).

However the wing hairs in the btsz$^{J5\text{-}2}$ homozygous tissue had distal orientation as in the wild type tissue. This indicated that btsz is not required for establishment of wing hair polarity but is involved in hair morphogenesis. To confirm this suggestion I decided to look for distribution of Flamingo, a core group protein forming proximal-distal domains, in btsz$^{J5\text{-}2}$ clones.

3.8 Btsz II Is Not Required For Cortical Polarization of Flamingo:

To check whether btsz II is required for cortical polarization of any of the core members, I looked for localization of flamingo in cells lacking btszII. Clones of cells lacking btszII were marked by lack of expression of CD2 and stained Fmi at 28 Hr apf using rabbit anti flamingo antibody.

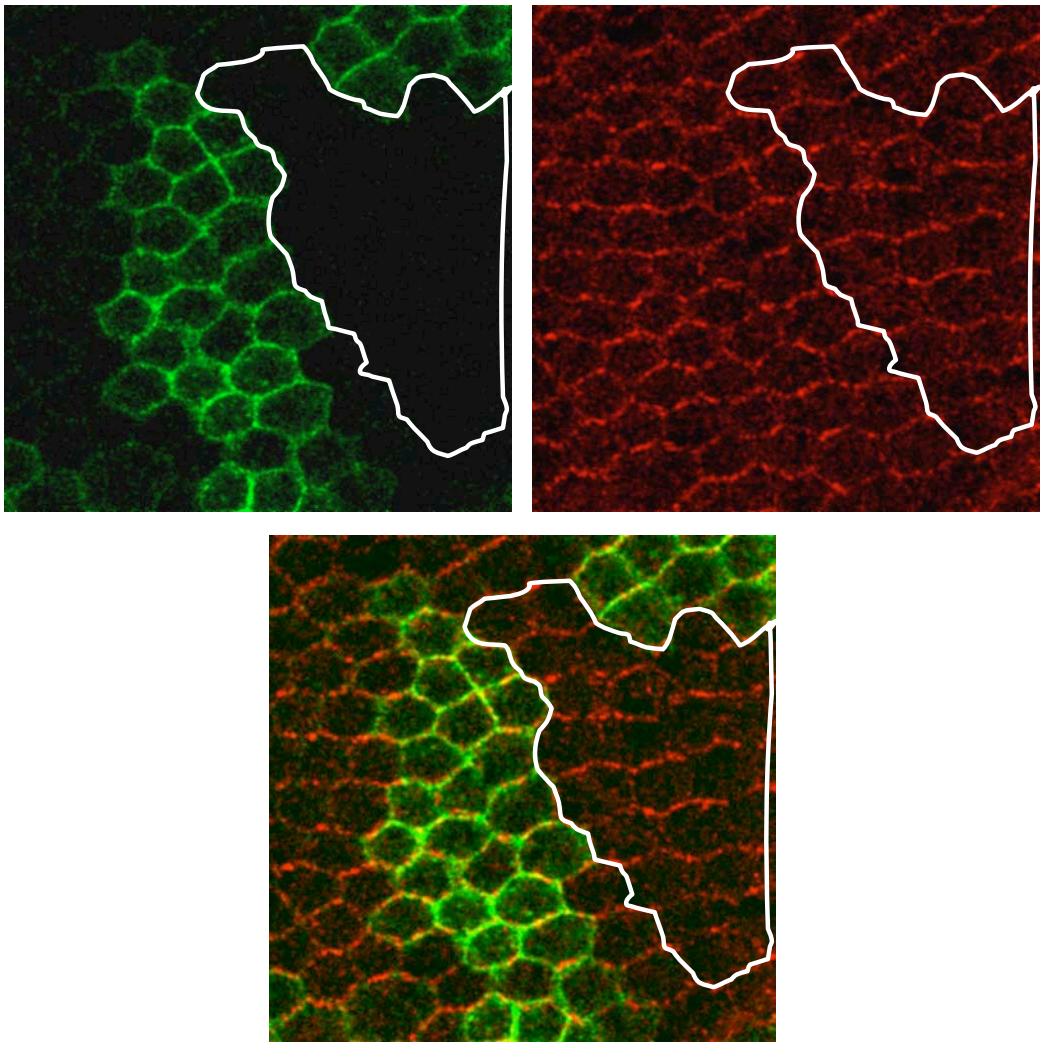


Fig. 22: Flamingo forms proximal-distal cortical domains in cells lacking btszII: Clones of $btsz^{J5\text{-}2}$ were induced at late 3rd instar stage. Pupae were collected 2nd day after induction of clones. Wings were fixed following PFA fixation protocol at 28 Hr apf stage. Btsz clones were marked with lack of CD2 expression. a) CD2 staining (green), the clonal area is marked with white boundaries. b) Flamingo (red) shows polarized distribution in wild type cells and in clonal cell too. c) Merge of (a) and (b).

3.9 btsz-II localization:

In absence of antibody recognizing the endogenous protein, I checked localization of myc tagged version of btsz-II described by Serano et al. The myc-btsz-II construct expresses Btsz-II isoform with Myc tag at the N terminus in a UAS-GAL4 dependent manner.

Myc-Btsz-II shows apical distribution and also marks the plasma membrane. Interestingly the cells over-expressing Btsz-II show accumulation of Wdb.

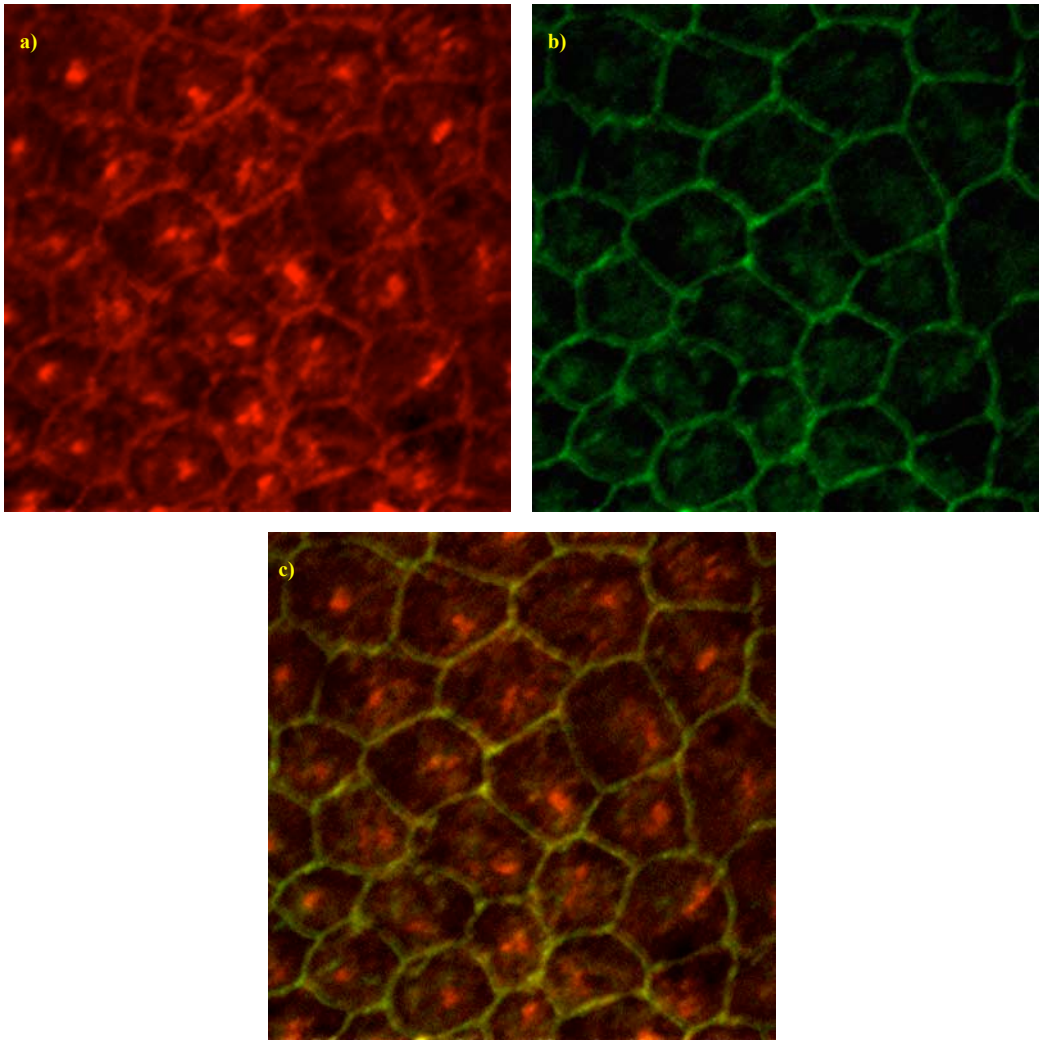


Fig. 23: Localization of Myc tagged BtszII: N-Myc tagged btszII was expressed in a UAS-Gal4 dependent manner using ApGal4. The wings were fixed with methanol at around 26hr apf. A single confocal section is shown here a) N-Myc Btsz II was marked with mouse anti myc antibody. b) Dcad c) Merge**.**

3.10 GFP-CT goes plasma membrane and is sufficient for hair duplication phenotype.

A transgenic fly line with N terminal GFP-tagged construct was used to over-express the C terminal region of btsz that contains the tandem C2 domains (GFP-CT) in a GAL4 dependent manner. Over-expression of this construct was found to be sufficient to cause wing hair duplication phenotype.

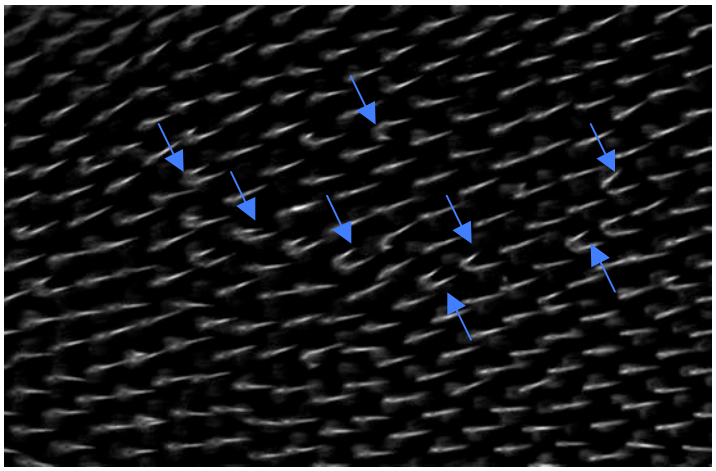


Fig.24: Over-expression of GFP-CT results in wing hair duplication phenotype: A dark field microscopic image of adult wing over-expressing GFPCT driven by Ap-Gal4. Blue arrows mark some of the hair duplications.

To check localization pattern of GFP-CT, the construct was expressed in the posterior compartment of the wing in Hh-Gal4 dependent manner. The wings were fixed following methanol fixation protocol in order to preserve the apical microtubule web. The apical junctions were marked with D-Cadherin. GFP-CT was found to localizing with the plasma membrane. Though no polarized distribution was observed (fig19), GFP-CT was found enriched apically. In addition to the membrane localization GFP-CT also showed some cytoplasmic distribution, but no vesicular or punctate pattern was observed. The cytoplasmic distribution was pronounced in the basal part of the wing epithelial cell than the apical most region (fig.20).

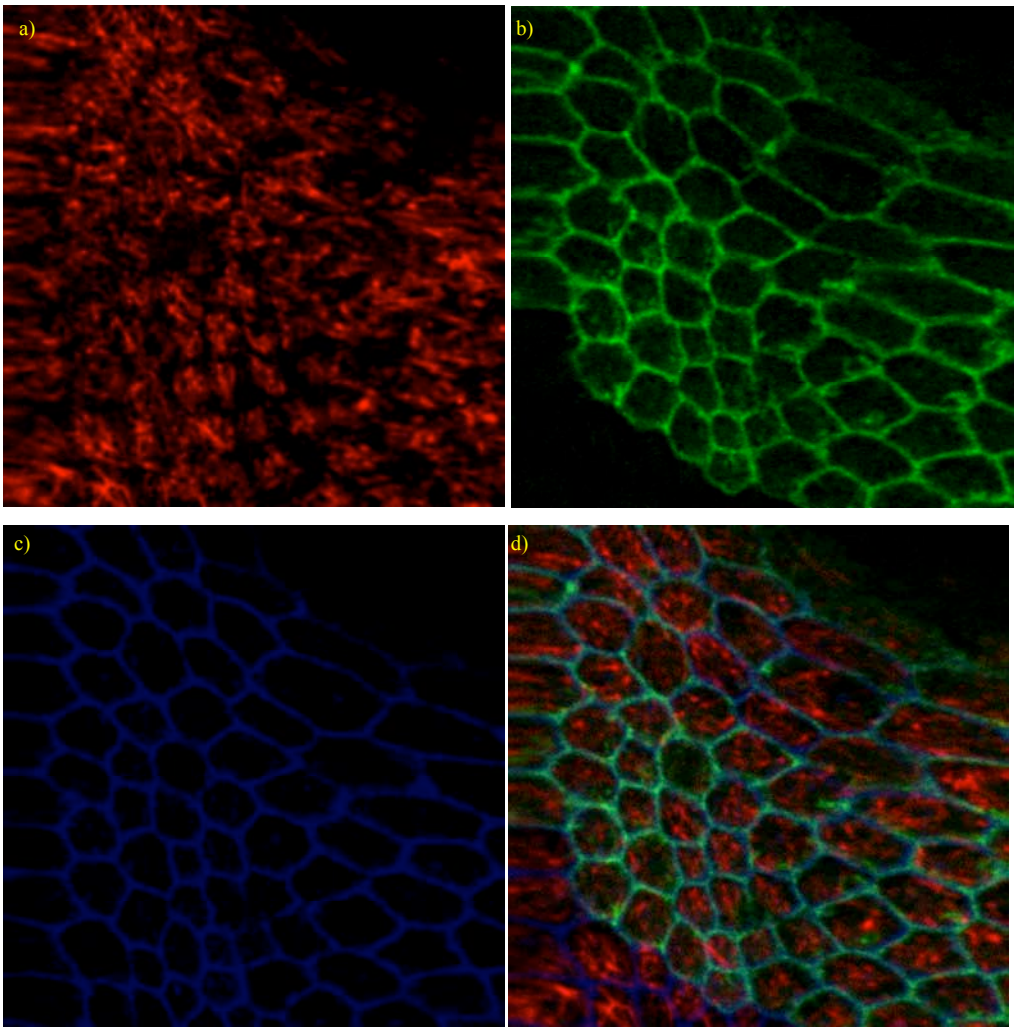


Fig.25: GFP-CT goes to plasma membrane: GFP-CT construct was expressed in the pupal wing epithelium in the posterior compartment with Hh-Gal4. The wings were fixed with methanol at 26-Hr apf to preserve the microtubules. Single confocal section is shown here a) α-Acetylated tubulin, b) GFP-CT, c) D-Cadherin marking the apical junctions. d) Merge.

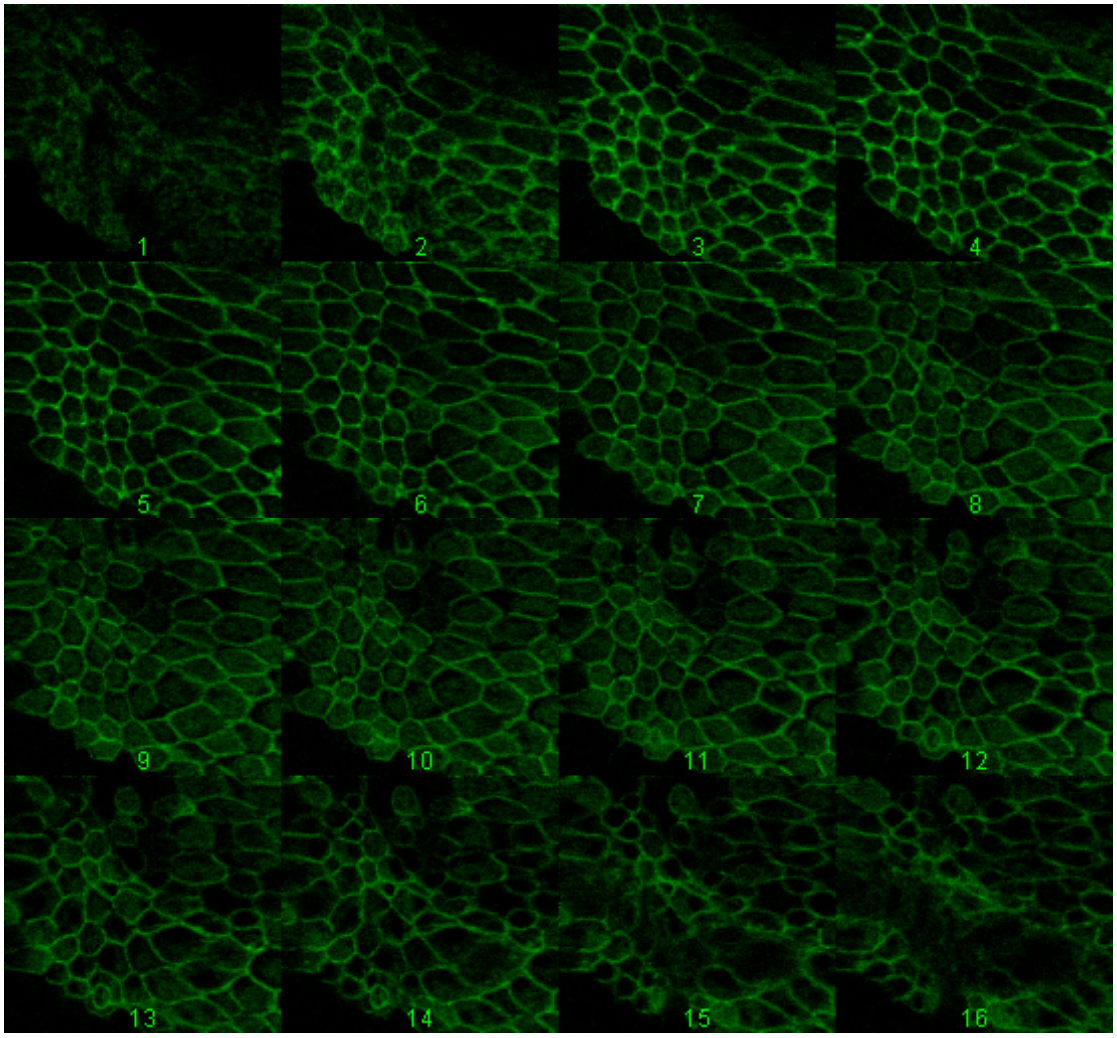


Fig. 26: Montage of confocal sections along the Z-axis showing GFP-CT localization pattern in the wing epithelium: The first section is apical most and the 16th section is basal most. A distance of 200nm separates the sections. The section no. 3 is shown in fig. 19.

Chapter IV: Discussion

The field of tissue polarity took the first big leap with discovery that Flamingo forms proximal-distal cortical domains. After that several other proteins were found to form either proximal or distal or proximo-distal cortical domains. Epistatic analysis established a core group of proteins forming cortical domains. The polarised distribution of the core proteins depend on each other and regulate the orientation of wing hair. However the core group members are not required for hair formation and are only required for maintaining hair polarity. So the machinery involved in hair formation and hair polarity are distinct.

The discovery of wdb provided a crucial link between these two sets of machinery since the expression of the dominant negative form of Wdb results in loss of regulation of both polarity and hair morphogenesis.

4.1 Motivation Behind The Yeast Two Hybrid Screen

The motivation behind this project was to find out how Wdb might be regulating both hair polarity and hair morphogenesis.

Wdb is component of the PP2A complex and acts as regulatory subunit of the tertiary complex. The active PP2A complex is made up a catalytic (phosphatase) subunit, a scaffolding subunit and a regulatory subunit. There are three subfamilies of the regulatory subunits with each subfamily having multiple isoforms. This huge diversity of the regulatory subunits itself speak of their significance. The regulatory subunits play very important role because of their ability to give substrate specificity to the catalytic (phosphatase) subunit of PP2A complex. This great variety of the regulatory subunits and the specificity offered by them makes PP2A a potent regulator of many cellular processes (Sontag E. 2001). Wdb is member of the B'/P56/PR61 subfamily and belong to the α type isoform.

Thus it is highly possible that Wdb interact with a variety of proteins to regulate various cellular processes. The dominant negative form of Wdb used for investigating

its role in planar cell polarity in the wing carried an N-terminal truncation. Over-expression of the dominant negative Wdb was found to result in absence of polarized distribution of Flamingo and defects in hair morphogenesis. A similar truncated construct of B56 regulatory subunit was shown previously to be able to bind with paxillin (substrate) and the catalytic subunit of PP2A, however the dephosphorylation of paxillin was far less effective (Ito et al., 2000).

Thus it can be argued that the defects in planar polarization and hair morphogenesis in cells over-expressing dominant negative Wdb might be due to absence or inefficient dephosphorylation of some specific substrates.

To understand how Wdb might be regulating planar cell polarity and wing hair morphogenesis it is important to identify either the genetic or protein-protein interactions involving Wdb. A genetic approach taken earlier by Hannus et al., had resulted in isolation of wdb mutants. Therefore I decided to look for direct protein to protein interaction and initiated a Yeast two Hybrid Screen in hope to find Wdb specific protein-protein interaction.

4.2 Wdb Interacts With Variety of Proteins

As expected the screen resulted in various different proteins interacting with Wdb. These included many novel proteins. Cyclin-G was one of the most abundantly isolated candidates with seven independent clones. PP2A regulatory subunits belonging to B' family are known to be interacting with Cyclin G (Okamoto et al., 1996). Thus isolation of Cyclin G in the screen provided validation.

However, the 18 proteins identified to be interacting with Wdb didn't include any of the previously studied established tissue polarity proteins. Out of 18, eight were novel proteins at the time of the screen. Later on CG9188 was identified as a Septin interacting protein 2 (Sip2), (Shih et al., 2002) and CG31306 was identified as bitesize. CG2467 is a protein with Zona Pellucida (ZP) like domain and a transmembrane domain. Among studied of ZP proteins in *Drosophila, dusky* and *miniature* complex is required for apical membrane reorganization during wing

differentiation (Roch et al., 2003) and *dumpy* is component of cuticle-epidermal cell attachment site (Wilkin et al., 2000). Functions of CG2467 remain to be investigated. Among other candidates CG3259 (dMIPT3) was identified as ortholog of Microtubule interacting protein T3 (MIPT3) based on sequence homology. The mammalian MIPT3 protein interacts specifically with TRAF3 (TNF receptor associated factor 3), and sequesters it to microtubule network in absence of activation (Ling L et al., 2000). There are two TRAF proteins in *Drosophila*, namely TRAF1 and TRAF2. Of these TRAF1 is known to be specifically interacting with Misshapen (Msn), (Liu H et al., 1999). Thus dMIPT3 might provide an indirect connection between Wdb and Msn.

Two independent clones of *tantalus* (CG8566), a protein involved in Sensory organ precursor (SOP) development were isolated (Dietrich et al., 2001). A single clone of Serendipity δ (*Sry δ*), a transcription factor was isolated. *Sry δ* is known to be involved in transcriptional regulation of bicoid, a maternal mRNA involved in oocyte patterning (Ruez et al., 1998, Payre et al., 1994).

Thus Wdb might be involved in regulation of variety of proteins involved in different cellular processes. As described in the results section I focused on *bitesize* due to the weak hair morphogenesis defects observed in btsz mutants and its genetic interaction with *wdb*. Thus, the protein-protein interaction and genetic interaction between wdb and btsz were positive considerations in addition to the hair morphogenesis defects observed in weak alleles of btsz.

4.3 CG31306/ bitesize Belong To The Synaptotagmin Like Protein (Slp) Family

All the predicted isoforms of bitesize contain two conserved tandem carboxy terminal C2 domains, also referred as CT (carboxy terminal) type C2 domains.

The C2 domain was originally identified as one of the four conserved (C1 through C4) domains in the α, β and γ isoforms of mammalian Calcium dependent protein kinase C isoforms (Nishizuka Y 1988). Later on C2 domains were found in several other proteins. Soon a family of proteins with two tandem carboxy terminal type (CT) C2 domains was identified. Most proteins with C2 domain function in signal

transduction pathways or membrane trafficking. This includes Synaptotagmin, Rabphilin (a Rab3A binding protein) and DOC2 a vesicular protein involved in exocytosis. Other than the PKC family and the CT family, C2 domains are present in a variety of proteins involved in GTPase regulation (rasGAP), proteins involved in lipid modification (PI3 kinases, Phospolipase C/PLC), tumor suppressor protein PTEN. Thus proteins with C2 domain are involved in a variety of cellular functions (Nalefski and Falke, 1996).

Recently a novel family with C terminal type C2 domains is described, the Synaptotagmin like protein (Slp) family (Fukuda and Mikoshiba, 2001). This family is characterised by absence of any transmembrane domain as present at the N terminus of synaptotagmin. Later on an N terminal SHD domain, Slp homology domain, was identified to be present in some members of the Slp family (Fukuda, Saegusa and Mikoshiba, 2001).

Bitesize essentially belongs to the Slp family due to presence of the carboxy terminal tandem C2 domains, the founding characteristic of Slp family. The *Drosophila* genome annotation release 3.1 predicts an isoforms with the SHD domain. But the attempts to isolate the isoforms with SHD domain have failed so far. This might be due to limitations of the methods used for cDNA search.

4.4 C2 Domains Are Versatile Lipid And Protein Binding Modules

The C2 domains vary in size from 100 to 130 residues and the structure of the C2 domains consists of a compact β sandwich of 8 anti parallel strands connected by variable top and bottom loops. The sequences involved in the β strands are more conserved than the loops. These variable loops provide pockets form the Ca^{2+} binding region (CBR). Many C2 domain proteins localize/translocate to membrane in a Ca^{2+} dependent manner and bind to the membrane phospholipids via the C2 domains. For this reason often the C2 domains are also referred as a Ca^{2+} dependent lipid binding domain (Rizo and Sudhof, 1998).

However, C2 domains are not obligatory Ca^{2+} and phospholipid binding domains, but can also act as protein interaction domain. The apical membrane targeting of Nedd4, a ubiquitin protein ligase E3, is mediated by its C2 domain via interaction with Annexin in a Ca^{2+} dependent manner (Plant et al., 2000). On the other hand some C2 domains bind membranes constitutively and do not bind Ca^{2+} at all. C2 domain of Rsp5, yeast homologue of Nedd4, binds specifically with phosphatydilinositols in a Ca^{2+} independent manner and targets the protein to perivacuolar endosomes (Dunn et al., 2004). Membrane localization of some C2 domains is Ca^{2+} independent as seen in the C2 domain of PTEN tumor suppressor protein. The C2 domain of PTEN binds specifically to anionic membranes and has a positive potential similar to that of the Ca^{2+} bound form of Syt1-C2A domain (Lee et al., 1999). In addition, the membrane association of PTEN is negatively regulated by phosphorylation of the C terminal tail region (Das et al., 2003).

In case of synaptotagmin the first C2A domain binds to syntaxin in a Ca^{2+} dependent fashion (Sugita et al., 1996) and the second C2B domain binds to clathrin adaptin (AP-2), (Zhang et al., 1994).

Thus C2 domains represent various mechanisms of Ca^{2+} dependent and independent membrane targeting and protein-protein interaction regulation.

4.5 The C2 Domains of Bitesize Lack The Conserved Aspartate Residues Present In A typical Ca^{2+} Dependent C2 Domain

The GFP tagged CT domain of btsz localize to the plasma membrane but does not show any polarised distribution. Five conserved aspartic acid residues that are present in the top loop of the C2 domain form the Ca^{2+} binding pocket in a typical Ca^{2+} dependent membrane localizing C2 domain. Sometimes Glu (E) residues replace these Aps (D) residues. The figure below shows Clustal multiple alignment of CT domains of various proteins. Of these the CT domain of Syt I is a well characterised Ca^{2+}

dependent CT domain. The five conserved aspartate residues involved in Ca^{2+} binding are highlighted with star.

As can be seen from the alignment, few of the five conserved aspartate residues are missing in both CT-A and CT-B domain of btsz. The CT-A and CT-B domain of btsz contain only four and three conserved Asp residues respectively.

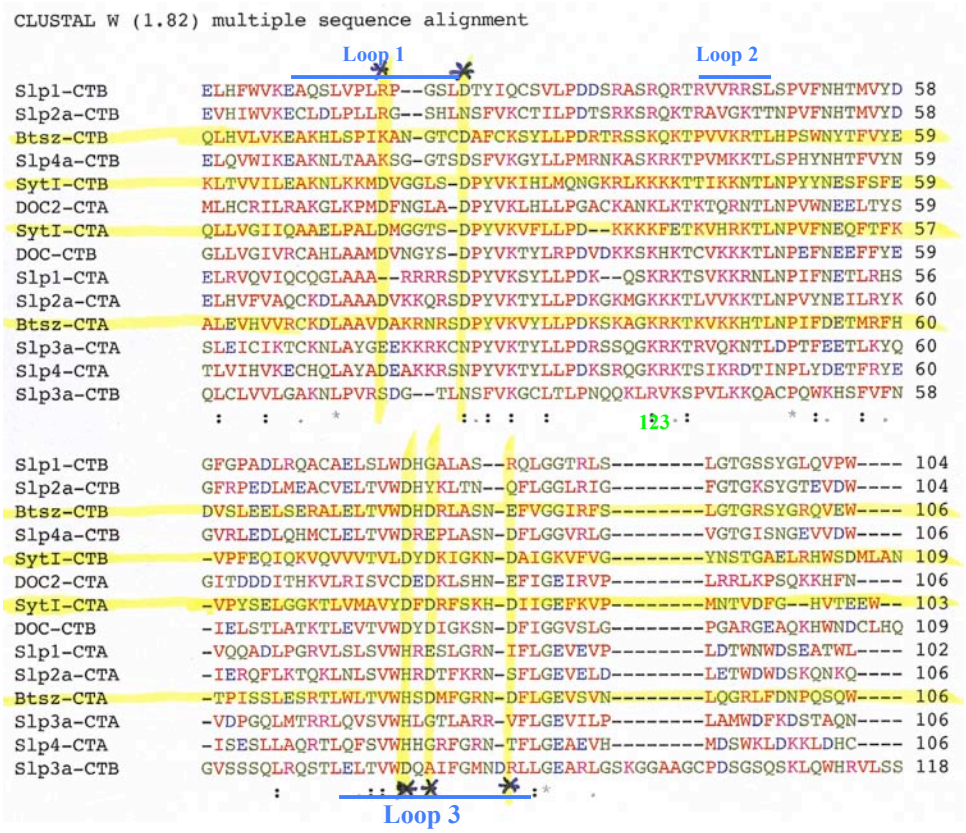


Fig 27: Clustal alignment of CT type C2 domains of Mouse SytI (Accession no. BAA07040), mouse SlpI (BAB32561), mouse Slp2a (BAB41082), mouse Slp3a (BAB41086), mouse Slp4/granuphilin-a (BAA84656), mouse DOC2 (BAA23430) and *D. melanogaster* Btsz-II (AAP04481). The regions forming the loops are marked with yellow line. The first and second C2 domains (CTA and CTB respectively) of btsz and Syt-I are highlighted in yellow. The position of the polybasic stretch is marked by numbers in green (123). The position of the five conserved aspartic acid residues involved in Ca^{2+} binding is marked by a star. Note that these residues are absent in btsz CTA and CTB.

A recent study of the C2 domain of Slp3 (Synaptotagmin like protein 3) shows that the CT domain of Slp proteins can behave in an atypical Ca^{2+} dependent manner

(Fukuda, 2002). The C2A domain of Slp3 binds to phospholiposomes in a Ca^{2+} dependent manner but contains only one Glu residue in the conserved Ca^{2+} binding pocket. Mutagenic analysis suggested that the tandem Glu resides (EE) in loop 1 and the stretch of polybasic amino acid residues (KRK) is required for Ca^{2+} dependent phospholipid binding. This indicates that Ca^{2+} dependent atypical C2 domains exist in the Slp family of which btsz is a member.

The subcellular localization of C2 domains correlates with their phospholipid binding specificity. PKCα and PKCγ translocate to plasma membrane rich in acidic phospholipids, in contrast the C2 domain of phospholipase A2 (cPLA2) localize to PC-rich nuclear envelope and endoplasmic reticulum (Gijon et al., 1999; Perisic et al., 1998). Thus it can be expected that the CT domain of btsz binds to the acidic phospholipids. However it is debatable whether the membrane localization of the CT domain of btsz is Ca^{2+} dependent or not and it will be interesting to know about any such dependence.

4.6 Planar Polarity Pathway Components Can Activate Ca^{2+} Influx In Vertebrate Embryos

Dsh is a multi-domain protein with DIX, PDZ and DEP domains and is component of both canonical and non-canonical Wnt signalling pathways (PCP). The DIX domain of Dsh is required specifically for Wnt signalling while the DEP domain is essential for the PCP signalling. The other non-canonical Wnt pathway, Wnt-Ca^{2+} signalling pathway, regulates gastrulation movements in vertebrate embryos through PKC activation in a G-protein dependent manner. The Wnt-Ca^{2+} pathway again utilize receptors belonging to the frizzled gene family (Kuhl et al., 2000).

Recently it has been shown that Dsh also functions in the Wnt-Ca^{2+} pathway to induce Ca^{2+} influx in Xenopus embryos in a G-protein independent manner (Sheldahl et al., 2003). The Dsh mediated induction of Ca^{2+} influx requires the DEP and PDZ domain and is independent of the DIX domain. This suggests the possibility that the

DEP domain of Dsh though not required for the canonical Wnt pathway may be involved in Wnt independent PCP pathway and the Wnt-Ca^{2+} pathway.

XDsh has been previously reported to transduce Wnt11/Fz7 signalling to affect convergent extension movements (Tada and Smith, 2000). It is a well established fact the several components of the PCP pathway are also involved in convergent extension. Prickle, another component of the PCP pathway is known to stimulate Ca^{2+} signalling in vertebrate embryos to regulate gastrulation movements (Veeman et al., 2003). Thus the data from studies of vertebrate gastrulation movements suggest that there is overlap between PCP and Wnt-Ca^{2+} pathways.

The Wnt-Ca^{2+} pathway is a G protein dependent pathway. There is enough evidence now that in *Drosophila* Fz acts a G coupled protein in both canonical signalling and PCP pathway and the fly Go subunit (Go) is required for Fz signalling (Katanaev et al., 2005).

With finding of involvement of a C2 domain protein (btsz) in regulation of wing hair morphogenesis and knowing that Dsh DEP domain is required for Ca^{2+} influx in vertebrate embryos during gastrulation movements, it will be interesting to know whether Ca^{2+} influx is required for hair morphogenesis. However, it needs to be established first whether C2 domain of btsz is an atypical Ca^{2+} dependent lipid membrane binding domain as seen in case of the C2A domain of Slp3a.

4.7 *bitesize* Positively Regulates Wing Hair Formation Process And The CT Domain of Btsz Is Sufficient For Ectopic Hair Formation

The loss of function of btsz-II results in truncated wing hair or bald patches. $btsz^{J5\text{-}2}$ is a P element excision allele that induces a frame-shift mutation resulting in a fragment with only the N terminal 393 amino acid region of btsz-II and lacks the C2 domains (Serano et al., 2003). This indicates that in absence of CT region btsz II in not efficiently active to elongate hair formation. Cells lacking btsz-II generate bald patches or stunted hair outgrowths. This difference may be due to the residual protein

received by the daughter cells from the mother cells. However it is also possible that btsz works in association with some other proteins and removal of btsz reduces the efficiency of the machinery involved in hair extension.

On the other hand gain of function (GFP-CT) over expression is sufficient to give hair duplication phenotype. This indicated that the C2 domains in the C terminus act as functional domains in hair morphogenesis and btsz is a positive regulator of hair morphogenesis.

The present study of localization of btsz is limited due to lack of antibody against endogenous protein. The over expressed myc-btszII protein shows mainly apical cytoplasmic distribution with weak cortical localization. In contrast, the GFP-CT shows strong cortical distribution. Probably this difference in level of cortical localization of GFP-CT and myc-btszII is reflected in stronger wing hair duplication phenotype caused by GFP-CT.

In absence of activity of btsz II the wing cells fail to extend the hair outgrowth and often result in truncated hairs. In the severe case the cell fail to form hair outgrowth all together, although the prehair is probably formed since the adult cells still show the mark of hair formation. This indicates that btsz is not required for the actin prehair formation but is involved in the process of extension of the prehair into a full hair outgrowth. In absence of proteins forming cortical domains, the wing hair is formed in centre of the wing as seen in case of Fz. However loss of btsz II did not affect the site of hair formation. Also, though GFP-CT construct shows cortical distribution, no proximal-distal polarity was observed. Thus it can be said that btsz do not play any role in deciding the sight of hair formation.

4.8 Possible Models For Regulation of Hair Morphogenesis By btsz

Regulating cortical localization of the btsz can be important to restrict formation of a single hair outgrowth per wing cell. One possibility is that the cortical localization of CT region of btsz is dependent on the phosphorylation status of btsz. There are examples from other proteins (like the CT domain of PTEN) of phosphorylation status

of the protein interferes the electrostatic membrane binding of the C2 domains. It should be noted here that *Drosophila* Rho associated kinase (Drok) is a negative regulator of hair morphogenesis. Drok acts downstream of Fz and its over expression suppresses the hair duplication phenotype caused by late over expression of Fz. Thus, it is possible that Drok and Wdb regulate the phopshorylation status of btsz and that in turn results in regulation of cortical association of btsz.

The extending hair is filled with actin-microtubule cytoskeleton and in addition also requires vesicular trafficking. In cells lacking btsz-II, though the hair growth is truncated, no reduction in cortical actin is seen. Thus btsz does not affect the actin cytoskeleton in general. The loss of function phenotype of btsz (truncated wing hairs/bald patches) and gain of function by over expression of CT domain is similar to that observed in case of the Misshapen, a Ser/Thr STE20 kinase. However, btsz is not required for maintaining wing hair polarity.

Among other proteins involved in regulation of hair morphogenesis, the Tricornered (Trc), a conserved Ser/Thr kinase belonging to Ndr family is also a positive regulator of hair morphogenesis. In absence of Trc, the hair morphogenesis process is delayed (He et al., 2005). Trc works in association with Furry and auto-phosphorylation of Trc at Ser281 is important for its kinase activity. In addition a Ca^{2+} dependent phophorylation site (Thr74) has been proposed to be important for Ndr1 regulation (Tamaskovic et al., 2003). The human Ndr1 protein interacts with S100B, a Ca^{2+} binding protein promoting neurite extension. This interaction is Ca^{2+} dependent and Ndr1 autophosphorylation activity depends on this interaction. Thus with knowledge of involvement of at least two putative Ca^{2+} dependent proteins in hair morphogenesis it will interesting to investigate whether and Ca^{2+} signalling plays any role in regulation of hair morphogenesis.

The process of hair morphogenesis in wing has two basic requirements. The first is the actin-microtubule cytoskeleton which fills the hair outgrowth and directed vesicular traffic for extending the hair outgrowth. In addition the machinery also takes care to avoid branching of the growing hair. In addition wing cells secrete cuticulin

early in process of hair outgrowth (Wong and Adler, 1993). Not much is known about regulation of cuticlulin secretion. However it not clear how to culticulin secretion is connected with hair extension.

The hair duplication phenotype is result of formation of two prehairs followed by efficient extension of the extra prehair. Both these process require various proteins acting down stream of Fz signalling. In cells lacking btsz-II no difference in cortical actin of observed, but the hair outgrowth was not robust. This suggests that btsz is involved in hair extension, the second stage of hair morphogenesis. However, the wing hairs were stunted, no branching of wing hairs was evident in cells lacking btsz-II. This is in contrast with loss of function of Trc and Fry, which result in split wing hairs and the duplicate/multiple wing hair phenotype caused by loss of function of mwh, and DRok. This suggests that these proteins work to antagonize btsz in hair morphogenesis. This is further evident in case of over expression phenotype of Drok which suppresses hair duplication phenotype caused by late over expression of Fz while over expression of GFP-CT results in hair duplication phenotype. Thus btsz do not belong to the group of genes regulating hair morphogenesis formed by *mwh, inturned, furry* and *Trc*.

However, the mechanism by which the C2 domain of btsz regulates hair extension is not known. In addition to their role as Ca^{2+} responsive membrane binding modules C2 domains same time can act as protein-protein interaction domains too. Thus it is possible that the membrane targeted C2 domain interacts with some cortical protein.

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